

MOLECULAR ARCHITECTURE OF THE HYPHAL WALL IN THE
WATER MOLD, *ACHLYA AMBISEXUALIS* RAPER

BY

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I would like to dedicate this dissertation to the memory of John R. Raper, who initiated physiological studies of *Achlya* by his pioneering research on the hormonal aspects of mating, and who, years later, stimulated my interest in fungal physiology and genetics.

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In order to elucidate the molecular architecture of the hyphal wall in *Achlya ambisexualis* Raper both chemical and morphological analyses were done. Isolated cleaned walls were fractionated chemically. Acid and enzyme hydrolysates of the resulting polymers or of the unfractionated wall were analyzed for their neutral sugar content and for their pattern of linkage and branching. Glucose was the only monosaccharide found, but three disaccharides were detected, laminaribiose, gentiobiose, and cellobiose, indicating the presence of β 1,3; β 1,6; and β 1,4 linkages. Close to 40% of the wall was found to consist of acid-soluble glucans of β 1,3 linkages with single β 1,6 linked glucose units every fifth monomer. A much lower percentage (7%) of the wall was soluble in alkali following the acid treatment. The structure of this fraction was determined to be a linear polymer of β 1,3 and β 1,4 linkages with occasional β 1,6 side chains. About 20% of the wall was solubilized by known cellulose solvents and was considered to be cellulose II based on x-ray diffraction analysis. Nearly 6% carbohydrate remained after these treatments. This insoluble residuum was found to have a linkage pattern similar to the alkali-soluble

fraction. Almost 3% of the wall was found to be glucosamine, most of which was in an insoluble form. After additional analysis it was concluded that this component was a weakly acetylated chitin. A 10% protein component was found in the wall, and amino acid analysis revealed the total spectrum of amino acids including hydroxyproline. Very small amounts of uronic acids and phosphorus were found, but virtually no lipid was detected.

Ultrastructural analyses of carbon-platinum surface replicas of hyphae treated either chemically or enzymatically, of isolated walls treated chemically, and of various wall fractions were performed. Both laminarinase, laminarinase plus protease, and acid plus alkali treatments removed the acid- and alkali-soluble glucans and revealed the underlying microfibrils of cellulose. The addition of cellulase to the laminarinase plus protease mixture resulted in virtual dissolution of the hyphae. Cadoxen, following acid and alkali treatments, resulted in almost total removal of the microfibrillar pattern. Observations of the surfaces of the various wall fractions indicated that the acid-soluble phase was amorphous, the alkali-soluble and insoluble residuum both faintly microfibrillar, and the cellulose II strongly microfibrillar. The cellulose I and chitin fractions were both uniformly microfibrillar. Morphologically, the hyphal wall of *A. ambisexualis* is similar to that of other Phycomycetes. Basically, the wall consists of an outer amorphous portion of β 1,3 and β 1,6 glucans which covers an inner microfibrillar component.

From these studies two models of the wall were designed. One is a molecular model which attempts to describe the molecular architecture of the wall. The other model is a diagram of the various wall components based upon both chemical and ultrastructural studies.

INTRODUCTION

Hyphal walls in fungi, as in other organisms, provide an invaluable function. In addition to protecting the protoplast from environmental damage, they also, by their rigid nature, aid the organism in maintenance of its characteristic morphology. Although rigid, walls are also pliant allowing the necessary morphological changes which occur with growth. They also play a role in cellular recognition between different organisms, as in host-parasite interactions and immune responses. Walls are considered to be an integral part of the living system (Preston 1979), perhaps as a single macromolecular entity (Keegstra *et al.* 1973). A number of studies have been made in an attempt to elucidate the molecular structure of the cell walls of various organisms. The common pattern which emerges is that of a rigid fibrillar structure embedded in and covered by an amorphous matrix. Pores, perhaps proteinaceous in nature (Wrathall and Tatum 1973), are thought to exist in the wall and to allow the passage of macromolecules (Farkas 1979).

In this study, the hyphal wall of the water mold *Achlya ambisexualis* Raper was analyzed both chemically and ultrastructurally, and a model of its molecular structure is proposed. Previous research on this organism has indicated a correlation between lateral branching and increased production and secretion of the enzyme cellulase (Thomas and Mullins 1969). It has been theorized that localized hydrolytic action by this enzyme "softens" or restructures the wall allowing branch initiation to occur (Thomas and Mullins 1969). A logical step in the study of the role

of this enzyme in branching and growth is an in-depth analysis of the "substrate," *i.e.*, the hyphal wall.

One question which this study attempted to answer is "What are the bonds of interest in the hyphal wall?" What aspect or aspects of the wall are responsible for its integrity? Early observations that lysis of *A. ambisexualis* hyphae occurred within two hours following the application of a cellulase led to the suggestion that the structural integrity of the hyphal wall resides directly in the cellulosic component or indirectly between the cellulosic and another component (Mullins 1979). This is in sharp contrast to Preston (1974a) in his analyses of higher plant walls, who states that the matrix portion of the wall is the key to its integrity. Another feeling, however, is that both components are necessary for the integrity of fungal walls (Bartnicki-Garcia and Lippman 1967; Sietsma *et al.* 1968, 1969; Hunsley and Burnett 1970; Tokunaga and Bartnicki-Garcia 1971). Whatever the case, the two functions of the wall, maintenance of hyphal morphology and plasticity to allow growth, must be borne in mind in the consideration of its molecular structure. One further point is the observation of Hunsley and Burnett (1970) of a more loosely arranged fibrillar structure at growing hyphal apices than in more distal or nongrowing regions. As branching occurs subapically, the conformation of the wall must be changed in order for this to occur and it may be here that cellulase or related hydrolytic enzymes play a key role.

LITERATURE REVIEW

The Organism, *Achlya ambisexualis* Raper

Achlya ambisexualis Raper belongs to the Class Phycomycetes, Series Biflagellate, Order Saprolegniales, Family Saprolegniaceae (Alexopoulos 1952). An alternate classification divides the Phycomycetes into two subclasses, one of which is the Oomycetes which have differentiated gametangia and to which *Achlya* belongs (Alexopoulos 1952). One of the distinguishing characteristics of the Saprolegniales is the possession of cellulosic cell walls, a situation not commonly found in the fungi. Fungi of the genus *Achlya* inhabit fresh water and generally form colonies around pieces of decaying plant and animal material. *Achlya ambisexualis* is filamentous and is made up of coenocytic hyphae surrounded by a rigid wall. A mass of hyphae is termed a mycelium. Septa, complete plates in this fungus, are formed only at the base of the reproductive structures or are sometimes found in aging mycelia. Vegetative growth occurs at the hyphal apices, also the area of sporangial formation. Sporangial spores are motile and biflagellate, one flagellum of the whiplash type and the other of the tinsel type. Sexual reproduction is by gametangial contact in which there is transfer of male gametes produced in an antheridium to female gametes or oospheres produced in a oogonium via fertilization tubes (Alexopoulos 1952).

Sexual reproduction is initiated and sequentially controlled by a series of diffusible hormones. Raper (1939a, b; 1940) first described

this hormonal mechanism in *Achylla*. The sequence is described as follows:

- (1) vegetative female hyphae secrete hormone A into the growth medium;
- (2) this hormone is taken up by the male hyphae and the result is the production of numerous lateral branches, termed antheridial hyphae;
- (3) the induced male now secretes hormone B, (4) which is taken up by the female and causes the induction of oogonial initials;
- (5) two additional hormones, C and D, were postulated to be involved in the attraction and appression of the antheridia to and onto the oogonium;
- (6) this is followed by septal delimitation at the antheridial tip and at the base of the oogonium to form the functional sexual organs. These two cells are the site of meiosis and gametogenesis. Fertilization takes place with the formation and inward growth of fertilization tubes from the appressed antheridia into the oogonium. The species name, *A. ambisexualis*, was chosen by Raper (1939a) to emphasize the presence of a wide range of sexual capacities among the various isolates, *i.e.*, pure female or pure male, or either male and female depending upon its mating partner.

Hormone A was chemically characterized by McMorris and Barksdale (1967) and renamed antheridiol. Several structures were proposed (Arsenault *et al.* 1968) and two isomers of one of the proposed structures were synthesized, one of which resembled natural antheridiol in its physical and biological properties (Edwards *et al.* 1969). Hormone B was similarly chemically characterized and renamed oogoniol by McMorris *et al.* (1975). Both antheridiol and oogoniol are steroids and are the best characterized sex hormones of this structure found in the plant kingdom.

Strain E 87, a pure male, was used in this research. The response by this strain to antheridiol has been examined biochemically. Both

antheridial branching and an increase in the secretion of cellulase into the wall and its subsequent release into the medium have been found as a result of antheridiol treatment. The role of cellulase has been postulated to cause localized wall softening allowing antheridial branching to occur (Thomas and Mullins 1969). Observations of lateral wall thinning at the sites of branch initiation gave support to the wall softening theory (Mullins and Ellis 1974). RNA and protein synthesis have been found to be required for both branching and cellulase production (Kane *et al.* 1973; Horowitz and Russell 1974; Timberlake 1976).

Higher Plant Cell Walls

Although the principal subject of this dissertation is the study of a fungal wall, some discussion of higher plant cell wall structure and growth is relevant. Albersheim and co-workers (Albersheim *et al.* 1973; Bauer *et al.* 1973; Keegstra *et al.* 1973; Talmadge *et al.* 1973; Albersheim 1974) have done the most recent and major work in this area and this has generated considerable interest (Monro *et al.* 1974, 1976). In terms of carbohydrate polymers, the higher plant cell wall is more complex than that found in fungi. The technical approach, however, for studying both the chemical and physical aspects of walls is similar.

In general, more detailed wall analyses have been done on primary walls rather than secondary. Primary walls are less differentiated and occur in plant cells which are still growing. The basic cell wall structure of a number of dicotyledonous plants has been analyzed and found to be basically similar (Albersheim *et al.* 1973). Where differences in structure occur, they are usually in the linkage, number, and types of attached residues which act as side chains. Such differences, for

example in the hemicellulose or pectic portion of the wall, result in changes in physical properties and thus biological function (Aspinall 1973). Pectic substances and hemicelluloses together with cellulose form the bulk of the primary cell wall of higher plants. The carbohydrate polymers of the plant cell wall are: (1) cellulose; (2) hemicellulose (xylans and glucomannans); (3) pectic substances [galacturonans, arabinans, galactans and/or arabinogalactans, and rhamnogalacturonans (Talmadge *et al.* 1973)]; (4) glycoproteins (Aspinall 1973).

Most of these studies employed cultured cells and involved isolating and cleaning the cell walls, followed by fractionation either by the use of purified hydrolytic enzymes, or alkali, urea, and mild acid (Albersheim *et al.* 1973; Bauer *et al.* 1973; Talmadge *et al.* 1973; Albersheim 1974). Monosaccharides, fractionated polysaccharides, and total wall polysaccharides were analyzed for their monomeric structure, type of glycosidic linkage, and anomeric configuration by a combination of gas chromatography-mass spectrometry methylation analysis.

Analyses of an endopolygalacturonase digest indicated that the pectic polysaccharide portion consists of a rhamnogalacturonan main chain with side chains of arabinans and galactans. The galactan has been postulated to serve as a bridge to the hemicellulosic portions of the wall (Talmadge *et al.* 1973). The hemicellulose portion of the wall is basically a xyloglucan polymer. This component consists of two fragments, one, a seven-unit sugar, and the other, a nine-unit one. In addition to xylose and glucose, small amounts of galactose and fucose are present in the larger fragment (Bauer *et al.* 1973; Albersheim 1974). It has been speculated that covalent linkages exist between the pectic polysaccharides and the hemicellulosic portion of the wall, while non-covalent linkages

link the cellulosic and hemicellulosic wall components (Bauer *et al.* 1973). The amount of xyloglucan is sufficient to cover, via the formation of hydrogen bonds, all of the cellulose fibrils (Bauer *et al.* 1973).

Lampport and Northcote (1960) reported the existence of a specific protein occurring in plant walls which contains an imino acid, hydroxyproline, which is usually found only in trace amounts in cytoplasmic protein. It was suggested that this protein might be responsible for cross linking various wall components and that wall extension might be caused by the enzymatic reduction of disulphide bridges (Lampport 1965) or at least by the lability of certain covalent linkages in this glycoprotein (Lampport 1970). Structural studies of hydroxyproline-rich glycopeptides have indicated a polypeptide backbone with oligoarabinose side chains (Lampport 1967, 1969). Subsequently a hydroxyproline-rich glycopeptide was isolated which contained galactose bound by the hydroxyl group of serine, and a tentative structure was devised consisting of a serine with an attached galactose and four hydroxyprolines each with four arabinose molecules (Lampport 1973). There are many questions concerning cell wall proteins; for example, what sugar is covalently bound, is there more than one structural protein in the wall, and to which component of the wall is the protein attached (Preston 1979)?

The only molecular model which will be considered in detail is that designed by Keegstra *et al.* (1973) based on the chemical analyses of sycamore cell walls. Briefly, the matrix of the cell wall includes the pectic substances, the proteinaceous component, and the hemicellulosic materials while the cellulosic portion makes up the microfibrillar region. Covalent cross linkages are postulated to hold the matrix together, while hydrogen bonds are responsible for cementing the cellulose portion and

for binding the cellulosic molecules to the xyloglucan component of the matrix. The hydrogen bonding is so extensive that it is considered to have strength comparable to the covalent linkages. In the model the glucose moiety of the hemicellulose component lies parallel to the axis of the cellulose fiber and is bonded by hydrogen bonds. Arabinogalactan chains lying perpendicular to the cellulose fibrils bind the hemicellulose to the pectic substances by glycosidic linkages and may also play a role in binding the hydroxyproline-rich protein (Albersheim *et al.* 1973). Taking all the bonds into account a rigid matrix is formed (Albersheim 1974).

Cell elongation, as envisioned in the model, occurs by the ability of the cellulose molecules to slide past each other, suggesting that certain bonds are labile. It is postulated that for nonenzymatic creep—the slow yielding of the cell wall under constant stress thought to be responsible for cell growth (Preston 1974a)—to occur, only four consecutive hydrogen bonds need to be broken and it is thought that these bonds exist between the xyloglucan chains and the cellulose microfibrils. The rate of creep can be enhanced by lowering the pH or raising the temperature. Thus auxin may act because of its ability to stimulate growth via the activation of a hydrogen ion pump (Keegstra *et al.* 1973). An alternative suggestion (Albersheim 1974) is that bond breakage and reformation is mediated enzymatically with the involvement of a hydrolase and a synthetase.

This model has been criticized on several points (Preston 1979; Monro *et al.* 1976). In addition to criticisms of the techniques used, Preston (1979) felt the binding of xyloglucan and cellulose is unlikely because of the highly branched nature of the hemicellulose. Stronger

criticism of the model comes from the work of *Monro et al.* (1974, 1976). The model, or working hypothesis, devised by these researchers differs from Albersheim's in several respects. A small amount of hemicellulose (30% or less) is thought to serve as a covalent bridge between the protein and the microfibrils. In addition, a fraction of the wall protein itself or in conjunction with a polysaccharide is felt to be covalently bound to the microfibrils. More hydrogen bonds are implied in this model, especially in the matrix. *Monro et al.* (1974, 1976) have suggested that the bonds controlling creep should be at right angles to the direction of elongation, which is not the case in the Albersheim model. The Albersheim model is an explanation for cell expansion rather than for cell elongation (Monro 1976). Longitudinal growth in the *Monro* model occurs in the hydrogen bonded matrix region by the shearing of these bonds and is independent of microfibrillar orientation. These authors state also that studies of the roles of synthetic and hydrolytic enzymes must be done in order to reach a better understanding of what occurs in cell enlargement.

Fungal Walls

In general fungal walls appear to be simpler in structure than those of higher plants, at least simpler in the types of sugar monomers present. Hyphal walls are described as complex microfibrillar systems, the microfibrils embedded in a matrix, generally made up of glucans, mannans, and galactans (Northcote 1963; Aronson 1965; Rosenberger 1976). The microfibrillar component is usually chitinous, but in a few cases is cellulosic. A commonly used analogy of the combination of the matrix

and microfibrils is that of reinforced concrete (Rosenberger 1976). Fungal walls consist generally of 60 to 90% polysaccharide; other components are uronic acids, protein, lipids, melanin (in some cases), polyphosphates, and inorganic ions. Carbohydrate-protein complexes are formed by ester, o-glycosidic, and glucosamine linkages (Sturgeon 1974). A few detailed studies on yeasts and dermatophytes have been made on wall glycoproteins and peptido-polysaccharides (Gander 1974). In general these compounds function as enzymes or recognition sites in mating type or host-pathogen relationships (Gander 1974) and do not appear to have a role in wall structure.

Ultrastructural studies depict the wall as existing in basically two layers. The outer layer is the matrix and the inner, nearest the plasma membrane, is the microfibrillar. The change in layers in the wall is gradual rather than abrupt (Bartnicki-Garcia 1973). In some structures there is a third layer, melanin, which lies outside the matrix. Basic wall form seems to be similar in the various taxonomic groupings of fungi, even though the chemical composition differs (Bartnicki-Garcia 1973).

A correlation exists between the chemical structure of the wall and the major taxonomic groups of fungi (Bartnicki-Garcia 1968). Eight wall categories were created and the various taxonomic groups were placed in the appropriate one. Members of the first two categories contain cellulose as the microfibrillar portion, but one has glycogen as the matrix and the other glucan. The Acrasiales belong to the former group, while the Oomycetes belong to the latter. Organisms of the third category have both cellulose and chitin microfibrils

and are represented by members of the Hyphochytridiomycetes. The fourth is known as the chitosan-chitin category and includes the Zygomycetes. The fifth and by far the largest group is the chitin-glucan one which includes the Chytridiomycetes, the Ascomycetes, the Basidiomycetes, and the Deuteromycetes. Most yeasts belong in the sixth category, the mannan-glucan one. Yeasts with carotenoid pigments are placed in the seventh category, the mannan-chitin one. The last category consists of polygalactosamine-galactan, and are represented by the Trichomycetes.

In terms of the various polymers found in fungal walls, a distribution pattern can be made (Rosenberger 1976). R-glucans with $\beta 1,3$ and $\beta 1,6$ linkages are found in most groups except the Mucorales, while S-glucans, $\alpha 1,3$ linked, are limited to the Ascomycetes and the Basidiomycetes. Cellulose is found in a few Phycomycetes, while chitin is more universal. Chitosan and a polysaccharide of galactosamine are found in the Mucorales, and the Ascomycetes and Hyphomycetes respectively. Polyuronides are known in the Mucorales. Protein, or at least the common amino acids, is found universally. Hydroxyproline is reported in those which have cellulose in their walls. A more recent study has noted the presence of hydroxyproline in the basidiomycete *Tremella* (Cameron and Taylor 1976), where chitin occurs.

By altering the metabolism of the cell wall constituents, a fungus can change its morphology (Dow and Rubery 1977). Such alterations may involve changing from a mycelial to a yeast form or the reverse, or changing to a reproductive, survival, or invasive mode. Studies with *Mucor rouxii* indicated that there are higher quantities of protein and mannose in the cell walls of yeast forms as opposed to mycelial

(Bartnicki-Garcia and Nickerson 1962; Bartnicki-Garcia 1968; Dow and Rubery 1977). Additional differences include the presence of weakly acidic polysaccharides in yeast walls and strongly acidic ones in mycelial walls (Dow and Rubery 1977). Quantitative differences in the chemical composition have been noted in the walls of the different structures, such as sporangial and hyphal, within a single organism (Bartnicki-Garcia and Reyes 1964; Bartnicki-Garcia 1968; Cole *et al.* 1979; Mendoza *et al.* 1979).

Fungal Walls, Chemical Structure

Before describing the chemical structure of fungal walls certain inherent shortcomings of studies of this nature will be discussed. The first problem can be stated simply by the questions, "what is a clean wall?" and "what is the method used for determining cleanness?" Walls cleaned with hot alkali appeared pure microscopically, and chemical analyses revealed that many covalently bound amino acids were released (Cameron and Taylor 1976). Are these components part of the wall structure? Loosely bound wall constituents may protrude into periplasmic space; are these inherent structural compounds? An isolated wall is quite a different thing from a wall which is part of a living system and this must also be borne in mind (Crook and Johnston 1962; Cameron and Taylor 1976). Enzyme degradation is a commonly used method for studying wall composition but there are drawbacks to this technique, such as the use of impure enzymes and rearrangements in wall architecture caused by partial digestion (Farkas 1979). The products of chemical degradation must also be viewed with reservation due to the lability of certain constituents (Talmadge *et al.* 1973). Bearing these thoughts

in mind, it becomes clear that the results of an analysis of wall composition must be regarded with circumspection (Cameron and Taylor 1976). This does not mean that tentative wall models cannot be drawn, but that they must be considered in the light of the above restrictions.

Basidiomycete Walls .

Detailed studies of isolated walls of *Schizophyllum commune* have been made over the last few years by Wessels and his group (deVries and Wessels 1972, 1973a, b; Wessels *et al.* 1972; Sietsma and Wessels 1977, 1979). A lytic enzyme preparation from *Trichoderma viride* grown on isolated *Schizophyllum* walls was found to be active against the known substrates chitin and R- and S-glucan, thus giving a clue to the identity of the wall components (deVries and Wessels 1972, 1973a, b). The most external portion of the wall in *S. commune* is a water soluble layer of mucilage. The basic structure of this component is a β 1,3 glucan backbone with single glucose units linked through the sixth carbon atom of every third glucose residue (Wessels *et al.* 1972; Sietsma and Wessels 1977). An alkali soluble α 1,3 linked chain, the S-glucan, lies adjacent to the mucilage and next to this is the alkali insoluble R-glucan which is similar structurally to the mucilage except that it is more highly branched (Sietsma and Wessels 1977). The R-glucan is closely associated with the chitinous portion, identified by x-ray diffraction studies, of the wall. A tentative model of the R-glucan complex was postulated in which covalent linkages were suggested between the chitin and the R-glucan portions (Sietsma and Wessels 1979). Exo- β 1,3-glucanase hydrolysis of the R-glucan followed by chitinase treatment yielded a compound containing N-acetylglucosamine, glucose, lysine, citrulline,

glutamate, and glucosamine. The model drawn from these data describes this portion of the wall as consisting of a linear chitinous chain which is linked to an R-glucan oligomer by a bridge containing lysine, citrulline, glutamic acid, glucose, and N-acetylglucosamine. In summary, it was found that hyphal wall fragments consisted of 67.7% glucose, 3.4% mannose, 0.2% xylose, 12.5% N-acetylglucosamine, 6.4% amino acids, and 3.0% lipid. The mannose and xylose monomers are associated with the S-glucan component.

Chemical analyses of the walls of *Tremella mesenterica* indicated the presence of xylose, mannose, rhamnose, and fucose in addition to glucose (Cameron and Taylor 1976). The amino acid content of these walls was studied and, as mentioned previously, hydroxyproline was found (Cameron and Taylor 1976). *Polystictus* and *Ustilago* walls have been analyzed and the above listed monosaccharides were found; galactose was also found in *Ustilago* walls (Crook and Johnston 1962).

Deuteromycete Walls

These are the imperfect fungi and some are known only by their asexual states (Alexopoulos 1952). Enzyme degradation studies of *Aspergillus oryzae* and *Fusarium solani* indicated the presence of chitin and β 1,3 glucans. Wall degradation did not occur unless both enzymes were present or unless there was a glucanase pretreatment, leading to the speculation that the wall consists of a chitin-containing core masked by the glucan (Skujins *et al.* 1965). Chemical analyses of the carbohydrate content of isolated walls from *Aspergillus* sp. and *A. niger* showed that 50 to 60% of the wall is carbohydrate of which 4.3% is mannose, 5 to 14% is galactose, and the remainder is glucose (Ruiz-Herrera 1967; Cole *et al.*

1979). The amount of chitin in both organisms is 15%. Studies of several species of *Penicillium*, *F. oxysporum*, and *Botrytis cinerea* revealed the same proportion and types of monosaccharides as found in *Aspergillus*, although mannose was not universally found (Crook and Johnston 1962; Pengra *et al.* 1969).

Total protein measurements revealed between 7 and 8% of the dry weight of the wall. Amino acid analyses were performed on both *Aspergillus* organisms and the usual spectrum was found (Crook and Johnston 1962; Ruiz-Herrera 1967; Cole *et al.* 1979). Hydroxyproline was not found. The lipid content of readily extractable and bound lipids was assayed for two *Aspergillus* species. Extractable lipids were found to be present as 7.3% of the wall while bound lipids varied between 7 and 12% depending on the study (Ruiz-Herrera 1967; Cole *et al.* 1979). Because some of the lipid could only be extracted after acid treatment of the walls, some of the lipoidal material present in the wall is probably complexed with structural polysaccharides and/or proteins (Ruiz-Herrera 1967). Ash was not found in the *A. niger* wall (Cole *et al.* 1979), but was found in that of *Aspergillus* sp. (4%) (Ruiz-Herrera 1967). Phosphorus content in both types of walls was found to be very low (0.1%) (Ruiz-Herrera 1967; Cole *et al.* 1979).

Ascomycete Walls

An analysis of the neutral sugars isolated from *Chaetomium globosum* and *Neurospora sitophila* indicated high glucose and low mannose and galactose amounts. Glucosamine was found in the walls of both organisms, and galactosamine was found in *Neurospora* (Crook and Johnston 1962). The presence of chitin was established by the usual means. Treatment of the

wall with exo- and endo- β 1,3-glucanases indicated the presence of a β 1,3 glucan with some β 1,6 linked glucose residues. From this datum it was proposed that the wall consists of layers of β 1,3 glucans overlying a chitinous core (Potgieter and Alexander 1965). It was also noted in this study that, although there was noticeable wall thinning after exhaustive enzyme treatment, the characteristic hyphal morphology remained unaltered (Potgieter and Alexander 1965).

More complete studies were performed on *Neurospora crassa* walls where analyses of wild type and single gene morphological mutants ("colonial") walls were performed. *Neurospora crassa* walls were separated into four fractions based on their solubilities in a variety of solvents (Mahadevan and Tatum 1965). Changes in fraction I, basically consisting of glucose, galactosamine, and glucuronic acids, were felt to be the predominant factor in influencing colonial morphology (Mahadevan and Tatum 1965). Higher amounts of uronic acids in wild type walls suggested that these compounds have a role in regulating linear hyphal growth, possibly due to the increased water content accompanying these compounds which may increase wall plasticity (Cardemil and Pincheira 1979). An increase in mannose and galactose in the mutants suggested that colonial morphology may result from higher levels of a branching mannan component allowing increased bonding and therefore more rigidity (Cardemil and Pincheira 1979).

Five peptide fractions extracted from *N. crassa* walls with weak alkali indicated the presence of all normally occurring amino acids (Wrathall and Tatum 1973). Quantitative differences were found, but there were similarities in the ratio of acidic to basic components and in the proportions of hydrophilic residues. O-glycosyl-serine linkages

were discovered which indicated that this component was part of a glycoprotein which did not appear to be covalently linked to any other major wall constituent. It was felt that this was evidence for a separate glycoprotein reticulum as a wall component, thus supporting the earlier work of Hunsley and Burnett (1970).

The yeast cell wall has been studied in great detail and the following will only briefly touch on the subject. Three fractions of the yeast wall were obtained by extractions with anhydrous ethylenediamine (Korn and Northcote 1960). Fraction A, soluble in water and ethylenediamine, contained the total spectrum of amino acids plus mannose and glucosamine (36% of the total amino sugar found). This fraction was felt to represent a mannan-protein complex with the amino sugar serving as a link between the polysaccharide and protein components (Korn and Northcote 1960). Fraction B, insoluble in water but soluble in ethylenediamine, was similar to A, except that glucose was present. Fraction C, insoluble in both solvents, contained 58% of the glucosamine in addition to chitin. Subsequent work has confirmed and extended these data. Both the glucan and mannan components have been characterized more completely. The major portion of yeast glucan is a β 1,3 linked polymer with some β 1,6 linkages and the minor portion is mainly β 1,6 linked with a few β 1,3 linked chains which may occur as interchain or interresidue linkers (Manners *et al.* 1973a, b). It was thought that these glucans provide a structural function with the β 1,3 component forming an inner fibrillar layer (Cabib 1975). Yeast mannan is a polymer of one protein and two carbohydrate moieties, and may have both immunological and structural functions (Cabib 1975).

Two models have been proposed for the yeast cell wall. In one model (Lampen 1968) the wall is made up of phosphomannans which are located in the outer layer of the wall. Wall-bound enzymes exist in this portion of the wall and release of these enzymes or cleavage of this fraction occurs by the action of an enzyme, the PR-factor, a "mannosidase." A smaller mannan is linked to the phosphomannan complex and also to glucan fibrils located in the inner portion of the wall. Protein molecules bound together by disulphide bridges make up part of the glucan portion of the wall. Observations by Kidby and Davies (1970) of enzyme release by sonication or thiol treatment in addition to previous studies by Bacon *et al.* (1965) led to a slight alteration of this model. In the altered model enzymes are inserted between the outer and middle layers and are held by non-chemical means. The structural integrity of the external wall layer is maintained by disulphide bridges. In this model the middle layer is a mannan-glucan associated with disulphide-linked proteins which are bound to a glucan chain which lies just outside the plasma membrane.

Phycomycete Walls

Initial wall analysis of *Allomyces macrogynus* (a uniflagellate Phycomycete) (Aronson and Machlis 1959) indicated the presence of chitin, glucan, ash, and protein, the latter depending, however, on the method of wall purification. Chemically cleaned walls contain 68% chitin, 8% glucan, and 10% ash, while walls cleaned with buffer and water contain 58% chitin, 16% glucan, 8% ash, and 10% protein. It is obvious that the two methods of cleaning resulted in modifications of the wall constituents. Amino acid analysis of a polypeptide fraction revealed a wide range of

these compounds (Youatt 1977). α 1,4 and α 1,6 linkages were found in the hyphal walls and β 1,3 in the walls of discharge plugs (Youatt 1977).

Rhizomyces sp., another uniflagellate form, has both cellulose and chitin in its walls as determined by x-ray diffraction studies (Fuller and Barshad 1960).

Zygomycete walls

Zygorhynchus vилleminii walls contain galactose, mannose, fucose (the most abundant monosaccharide found), and glucosamine plus the usual assortment of amino acids (Crook and Johnston 1962). Two acidic polysaccharides, mucoran and mucoric acid, were isolated and analyzed from *Mucor rouxii* (Bartnicki-Garcia and Reyes 1968). Mucoran is made up of 2 fucose:3 mannose:5 glucuronic acid and mucoric acid is a homopolymer of glucuronic acid. It was felt that these components made up a single heteropolymer (Bartnicki-Garcia and Reyes 1968). Later studies of the walls of *M. mucedo* revealed a glycuronan made up of 5 fucose:1 mannose:1 galactose:6 glucuronic acid non-covalently bound to glucosamine polymers (Datema *et al.* 1977a). The homopolymeric glucuronic acid part of the isolated glycuronan is thought to be associated with the glucosamine polymers (Datema *et al.* 1977a). *Mucor* walls also contain weakly acetylated chitin, chitin, and chitosan (Bartnicki-Garcia and Nickerson 1962; Bartnicki-Garcia 1968; Datema *et al.* 1977b). On a percentage w/w basis the composition of the hyphal wall of *M. mucedo* is 7% neutral sugar, 12% uronic acid, 16% phosphate, 32% hexosamine, 13% protein, 10% amino acids, and 13% ash (Datema *et al.* 1977a, b).

Oomycete walls (Leptomitales)

Analyses of buffer-water washed sonicated walls of *Sapromyces elongatus* indicated a typical Oomycete wall, containing 91% glucan,

4% protein, and 0.1% ash with glucose as the only monosaccharide (Pao and Aronson 1970). β 1,3, β 1,4, and β 1,6 linkages were found. Weakly crystalline cellulose I was present but chitin was not detected (Pao and Aronson 1970). The walls contained the full complement of amino acids with aspartic acid, glutamic acid, serine, and threonine the most abundant (46% of the wall protein). Hydroxyproline is 2.5% of the total amino acid content. No lipids were found. *Apodachlya* sp. and *A. brachynema* walls differ from those of *Sapromyces* in having glucosamine (Sietsma *et al.* 1969; Lin *et al.* 1976). X-ray diffraction studies and stains for chitin indicated the presence of both weakly crystalline cellulose I and chitin in these walls (Lin and Aronson 1970). The walls of *A. brachynema* contain phospholipids, fatty acids, and triglycerides (Sietsma *et al.* 1969). Linkage studies of *A. brachynema* revealed that 4% of the dry weight of the wall is soluble in Schweitzer's reagent and consists solely of β 1,4 linkages, and 52% was found to be a branched β 1,3 and β 1,6 linked glucan, and 32% a linear β 1,3 linked glucan (Sietsma *et al.* 1968). *Apodachlya* sp. walls contain 67% total glucose, 18% chitin, 9% cellulose, 6.4% protein, 1.5% acid-soluble hexosamine, and 3.1% alkali-soluble hexosamine (Lin and Aronson 1970; Lin *et al.* 1976). Analysis of the hyphal wall chemistry of *Leptomitus lacteus* indicated similarity to the walls of *Sapromyces* and *Apodachlya*, especially in regard to the linkage pattern (Aronson and Lin 1978).

Oomycete walls (Peronosporales)

Wall chemistry analyses of a number of species of *Phytophthora* indicated the presence of 90% glucan, 4% protein (10% reported in one species), 2% lipid, 0.4% phosphorus plus small amounts of mannose, glucosamine, arabinose, xylose, galactose, rhamnose, ribose, and

galactosamine (Bartnicki-Garcia 1966; Bartnicki-Garcia and Lippman 1967; Novaes-Ledieu *et al.* 1967; Tokunaga and Bartnicki-Garcia 1971). Weakly crystalline cellulose I makes up about 25% of the wall (Novaes-Ledieu *et al.* 1967). About 5% of the total amino acid content is hydroxyproline (Bartnicki-Garcia 1966). Walls of various *Pythium* species have also been characterized and similar compositions have been reported, although the total glucan and cellulose is lower (82% and 20% respectively) and the lipid is higher (8%) (Cooper and Aronson 1967; Novaes-Ledieu *et al.* 1967; Sietsma *et al.* 1969). All the common amino acids including hydroxyproline have been reported (Novaes-Ledieu *et al.* 1967). Low levels of chitin have also been found (Dietrich 1973).

The same type of linkage pattern was found in *Phytophthora* and *Pythium* walls as was described for the Leptomitales (Bartnicki-Garcia 1966; Bartnicki-Garcia and Lippman 1966, 1967; Aronson *et al.* 1967; Cooper and Aronson 1967; Novaes-Ledieu *et al.* 1967; Eveleigh *et al.* 1968; Novaes-Ledieu and Jiménez-Martínez 1969; Sietsma *et al.* 1969, 1975; Zevenhuisen and Bartnicki-Garcia 1969; Tokunaga and Bartnicki-Garcia 1971; Yamada and Miyazaki 1976). The basic pattern which emerged from a number of studies is that of a highly branched glucan of β 1,3 and β 1,6 linkages covering and firmly bound to a β 1,4 linked linear glucan (cellulose). There are varying opinions as to the degree of branching and as to which linkage groups serve as main chains and which as branches (Eveleigh *et al.* 1968; Novaes-Ledieu and Jiménez-Martínez 1969; Sietsma *et al.* 1969, 1975; Zevenhuisen and Bartnicki-Garcia 1969; Yamada and Miyazaki 1976). Both components, the branched glucan and the cellulosic, are reported to be slightly contaminated by linkages of the other (Novaes-Ledieu and Jiménez-Martínez 1969; Zevenhuisen and Bartnicki-Garcia 1969;

Sietsma *et al.* 1975). A β 1,2 glucan was reported for the walls of one species of *Pythium* (Mitchell and Sabar 1966) and an α 1,3 glucan was reported for a species of *Phytophthora* (Miyazaki *et al.* 1974).

Oomycete walls (Saprolegniales)

The basic pattern which has been described for the Leptomitales and the Peronosporales is also seen in the Saprolegniales, the principle differences lying in the relative quantities of the various components. The walls of four species of *Saprolegnia* have been analyzed. The predominant monosaccharide of *S. ferax* is glucose while considerably smaller amounts of glucosamine, mannose, rhamnose, and ribose have been found (Crook and Johnston 1962; Parker *et al.* 1963; Novaes-Ledieu *et al.* 1967). Quantitative studies revealed 93 or 85% total carbohydrate, 3 or 1.1% protein, 1.7 or 2.7% hexosamines, and 1 or 5% lipids depending on the study (Novaes-Ledieu *et al.* 1967; Sietsma *et al.* 1969). All the studies indicated that cellulose is present in these walls; however, there are vast quantitative differences ranging from 42% (Novaes-Ledieu *et al.* 1967) to 18% (Sietsma *et al.* 1969) to 15% (Parker *et al.* 1963). In one study an attempt was made to estimate the proportion of linkages and it was found that 18% are β 1,4, 44% are branched β 1,3 with β 1,6 linkages, and 20% are linear β 1,3 (Sietsma *et al.* 1969). The usual amino acid composition was found (Crook and Johnston 1962; Novaes-Ledieu *et al.* 1967). Other species of *Saprolegnia*, *S. litoralis*, *S. monoica*, and *S. diclina* reveal essentially the same pattern, although uronic acids were reported in *S. litoralis* (Parker *et al.* 1963) and *S. diclina* (Cameron and Taylor 1976). Some quantitative differences were found in *S. diclina* possibly reflecting differences in wall preparation. These

walls consist of 72.6% neutral sugars, 0.9% amino sugars, 3% uronic acids, 8.5% protein, and 12% lipid (Cameron and Taylor 1976).

Wall chemistry of *Achyla flagellata*, *A. racemosa*, *A. ambisexualis*, *Brevilegnia unisperma* var. *delica*, *B. bispora*, *Dictyuchus sterilis* and *Dictyuchus* sp. is similar to *Saprolegnia* in all respects (Parker *et al.* 1963; Sietsma *et al.* 1969). The general linkage pattern common to all these fungi was established for *A. ambisexualis* and *D. sterilis* (Aronson *et al.* 1967; Sietsma *et al.* 1969). An attempt to determine the proportion of the linkages was made for *D. sterilis* and was found to be similar to that of *S. ferax* (Sietsma *et al.* 1969). Dietrich (1973), studying four Oomycete genera, found hexosamine in all the walls (the three *Achlya* species studied had the highest content: 2.4, 3.1, and 3.8%) and upon treatment of these walls with snail gut enzyme, N-acetylglucosamine at 1 and 2% levels was obtained. These results led to the speculation of the presence of a chitin/chitosan component in these heretofore considered chitinless walls (Dietrich 1973). An indirect indication of the presence of chitin in *Achyla* walls stems from the observation of Wang and LéJohn (1974) of the absence of a UTP requirement for glutamic dehydrogenase; UTP has been found to be necessary for activation of the enzyme in organisms with chitinless walls. The walls of the marine fungus, *Atkinsiella dubia*, have also been studied and in general these walls are similar to those described above (Aronson *et al.* 1967; Aronson and Fuller 1969). Notable differences are the protein content (13.7% in these fungi) and a very high level of hydroxyproline (20.4% of the total amino acid content and 2% of the dry weight of the wall) (Aronson and Fuller 1969).

Fungal Walls, Physical Structure

The only polymers whose structures will be described here are those which occur in Oomycete walls, *i.e.*, those with $\beta 1,3$; $\beta 1,4$; and $\beta 1,6$ linkages. Cellulose has been studied extensively. Glucose units in cellulose are joined by $\beta 1,4$ glycosidic bonds and it is because of this type of linkage that the polymer can be described as a flat ribbon (Rees 1977; Preston 1979). The chain is stabilized by hydrogen bonds which form between the third carbon of a glucose molecule and the ring oxygen of the adjacent glucose unit (Preston 1979). Each ribbon-like chain has numerous potentially hydrogen binding hydroxyl groups along each edge, so when two chains come in contact many hydrogen bonds form creating a stable structure (Preston 1979). In native cellulose, usually termed cellulose I, it is thought that the chains lie parallel with each other and parallel to the surface of the wall in staggered layers. In regenerated cellulose, usually termed cellulose II, the chains are antiparallel and lie in regularly stacked layers (Rees 1977; Preston 1979). The highly ordered arrangement of chains, known as a microfibril, creates a crystalline structure amenable to x-ray diffraction and polarizing light microscope studies. A microfibril is described as containing a central crystalline core (5 to 7 nm wide in higher plants) surrounded by a paracrystalline cortex. The cortex is made up of molecular chains lying parallel to the microfibril length. The chains, however, are not in a crystalline arrangement because of their mixed cellulose and hemicellulose content (Preston 1974a). With the cortex added to the core, the width of the microfibril is about 10 nm (Preston 1974a). It is felt that the chains in the cortex become increasingly

more hemicellulosic with increasing distance from the core (Preston 1974a).

Polymeric glucan chains linked together by β 1,3 bonds exist in a hollow helical pattern (Rees 1977; Preston 1979). Three suggestions have been made as to how the hollow area is filled: 1) by the formation of an inclusion complex with appropriately sized molecules; 2) by the formation of a double or triple helix with other β 1,3 linked chains, and 3) by the nesting of a number of β 1,3 linked chains (Rees 1977). The structural importance of these polymers is their ability to twist around each other forming a network which is effective in entangling other polysaccharides or in itself creating a strong, but flexible, assemblage of molecules (Rees 1977; Preston 1979). The helical conformation will also exist, even if the β 1,3 bonds are interrupted by β 1,4 bonds (Preston 1979).

Loosely jointed linkages and chains are formed when glucans are held together by β 1,6 bonds. There is a lot of freedom of rotation of molecules involved in this type of bonding because of the separation of the monomeric units by three bonds rather than two, placing the sugar rings further apart (Rees 1977). Commonly individual glucans such as these are not found in nature. Instead these glucans exist as branches on other types of polysaccharides (Rees 1977). It is speculated that the flexibility of these linkages may allow the molecules involved to aid in various biological interactions such as the entry and exit of enzymes (Rees 1977).

Fungal Walls, Morphology

There is general agreement that hyphal walls consist of an inner microfibrillar core of randomly oriented chitinous or cellulosic fibrils

covered by an amorphous matrix of varying chemical constituency (Aronson and Preston 1960). The walls of sporangial, spore, and the sexual apparatus may differ either by having an outer microfibrillar component or an outer melanin layer (Tokunaga and Bartnicki-Garcia 1971; Hegnauer and Hohl 1978; Cole *et al.* 1979; Hawes 1979; Mendoza *et al.* 1979).

Information on the morphology of hyphal walls, treated chemically and enzymatically, comes from ultrastructural studies of surface replicas and thin sectioned material. The classic study of wall morphology is that of Hunsley and Burnett (1970) who compared walls after sequential enzyme treatments of three different fungi, each representing one of the major taxa. Live hyphae were used for two reasons: 1) any artifacts brought about by wall isolation were eliminated; and 2) confidence that enzymatic digestion occurred from the outside in. Models for each of the three major groups were then developed from these data.

Walls of *Schizophyllum commune*, the representative Basidiomycete, have a four-layered structure which none of the three enzymes, laminarinase, pronase, or chitinase, could hydrolyze. However, preliminary treatment with KOH was effective in removing the outer protective (S-glucan) layer. Subsequent treatment with laminarinase revealed a microfibrillar outline which was clarified by the addition of pronase. Chitinase treatment following that of laminarinase and pronase removed the microfibrillar component. The model which was derived from this study depicts the wall as having an outer S-glucan layer bounded internally by R-glucan. The R-glucan is bordered on the inside by a thin, but discrete, proteinaceous sheet which in turn is complexed and intermixed with the chitinous microfibrils (Hunsley and Burnett 1970). Van der Valk and Wessels (1977) using

isolated walls did a similar study and found that pronase had no effect on the R-glucan-chitin portion of the wall. This led to the belief that there is no protein layer and no protein-chitin complex. Carbon-platinum replicas of the S-glucan revealed a surface composed of randomly oriented parallel arrays of short rodlets and a filamentous surface of the mucilage (Wessels *et al.* 1972).

Neurospora crassa is the representative Ascomycete which Hunsley and Burnett (1970) studied. In thin section the wall appeared three-layered. Laminarinase treatment removed the outer amorphous layer revealing a coarsely stranded network, more clearly resolved by the addition of pronase, filled with a matrix material. Chitinase in conjunction with the other two enzymes resulted in dissolution of the wall. Neither chitinase nor pronase added alone or in sequence had any effect at all. The model derived from these data envisions the wall as having an outer layer of β 1,3, β 1,6 glucan with an inner layer of protein in which is embedded coarse strands of a glycoprotein (glucan-peptide-galactosamine) reticulum. A discrete protein layer lies between the reticulum and the chitinous microfibrils which are embedded in a protein "matrix" and lie in the innermost part of the wall (Hunsley and Burnett 1970). A similar study by Mahadevan and Tatum (1967) indicated that the wall consists of an outer coarse fibrillar layer (glucan-peptide-galactosamine) and an inner layer of primarily β 1,3 glucan with an embedded core of fine chitin fibrils.

Phytophthora parasitica walls were studied in order to derive a model for a Phycomycete wall. The two-layered wall has a finely granular amorphous surface which is unaffected by treatment with cellulase or

pronase or both. Laminarinase treatment resulted in the exposure of microfibrils whose outlines were more pronounced if pronase treatment followed. Laminarinase and cellulase treatment resulted in almost total digestion. The model of the wall designed from these studies describes an outer amorphous layer of β 1,3; β 1,6 linked glucan and an inner layer of cellulose embedded in protein. Similar layering has been seen in the hyphal walls of *Phytophthora palmivora* and *Pythium acanthicum* (Tokunaga and Bartnicki-Garcia 1971; Sietsma *et al.* 1975; Hegnauer and Hohl 1978). Chemical removal of the outer amorphous layer of isolated walls of *Saprolegnia littoralis* and *Atkinsiella dubia* and chemical and enzymatic removal of this layer in *Sapromyces elongatus* revealed a distinctly microfibrillar layer (Parker *et al.* 1963; Aronson and Fuller 1969; Pao and Aronson 1970).

Isolated walls of *Choanephora cucurbitarium*, another Phycomycete, were found to exist in two layers, an outer thick layer of randomly oriented microfibrils made up of a mixture of chitosan, protein, and lipids, and an inner thin layer of chitinous microfibrils oriented in a parallel fashion (Letourneau *et al.* 1976). Microfibrillar orientation of the hyphal walls of *Linderina pennisporea* is longitudinal except in the most interior portion of the wall where it is random (Young 1970). Sporangiphore walls in this same organism are similar except for the existence of spicules covering the outer surface of the wall (Young 1970).

Fungal Walls, Growth

Burnett (1968) presented a diagram of his views of apical and subapical wall organization and how it is altered in response to growth.

The hyphal tip is thin-walled and non-extensible, but the area directly behind the tip is thicker-walled and it is here that maximum intussusception takes place. Distal to this zone lies a second thick-walled area known as the region of maximum extensibility. In the most distal region described, the wall reaches its maximum thickness and becomes rigid. It has been suggested that the subapical wall in *Phytophthora parasitica* has more protein in which the microfibrils are embedded and a greater degree of microfibrillar aggregation than is found in the apical (Hunsley and Burnett 1970), which may account for the increasing rigidity of this part of the wall. In this most distally described area of the wall the arrangement of the microfibrils is longitudinal as compared to the transverse arrangement nearer the tip. Growth is explained by a change in the balance between synthetic and lytic enzymes (Bartnicki-Garcia 1973) which allows for turgor driven apical expansion (Thomas 1970; Bartnicki-Garcia and Lippman 1972).

Autoradiographic studies indicated that the sites of growth are at the tip (Van der Valk and Wessels 1977), although some wall thickening and modification is seen subapically (Bartnicki-Garcia 1973). It has been hypothesized that vesicles play a role in wall synthesis based on the observation of their accumulation at growing tips (Heath *et al.* 1971; Van der Valk and Wessels 1976; Beakes and Gay 1978; Hawes 1979) and at the sites of antheridial initials (Mullins and Ellis 1974). The suggestion has been made that these vesicles carry wall degrading enzymes (Mullins and Ellis 1974; Fèvre 1977) and materials for plasmalemma and wall synthesis (Bartnicki-Garcia 1973).

MATERIALS AND METHODS

Organism and Culturing Techniques

Strain E 87 male of *Achlya ambisexualis* Raper (Barksdale 1960) obtained from Dr. J. T. Mullins was the organism used in this study. Mycelia were grown on defined media (Mullins and Barksdale 1965; Kane 1971) on agar plates or in liquid culture on a reciprocating shaker (100 rpm) at 25°C. Two day old mycelium, grown on agar, was sporulated in 0.5 mM CaCl_2 on a reciprocating shaker (100 rpm) for 20 hr at 25°C with one change of solution after the first 2 hr. An inoculum of 200 000 zoospores was added to 20 ml of defined liquid medium and grown for 48 hr. Mycelium was harvested by vacuum filtration and washed two times with either 0.05 M potassium phosphate buffer pH 7.0 or 0.1 M tris-HCl buffer pH 7.5 depending on the subsequent method of hyphal wall preparation. Harvested washed mycelium was quick frozen at -70°C in a Revco Ultra Low freezer.

Hyphal Wall Isolation and Purification

In all cases hyphal walls were isolated by grinding in a chilled mortar and pestle 10 gm fresh weight frozen mycelial lots until a fine powder was obtained. The entire procedure was performed at 0-4°C. The appropriate buffer (final amount 20 ml) was added and grinding was continued. The resultant "slush" was centrifuged (Sorval RC-2B Automatic Refrigerated Centrifuge) at 1085 x *g* and the pellet was saved for further purification.

Two methods of cleaning hyphal walls were followed. In one, the walls were cleaned chemically by a modification of Tokunaga and Bartnicki-Garcia (1971) and, in the other, they were cleaned by repeated washings with buffer and water (Lin *et al.* 1976). In the first method the pellet was washed with phosphate buffer and then sonicated in 10 ml 2% sodium lauryl sulfate for one minute at 15 watts (Heat Systems-Ultrasonics Sonifier Cell Disruptor, Model W 185, fitted with a standard microtip). After sonication the suspension was placed in a 90°C water bath for 30 min. It was then centrifuged and the pellet was treated with 60 ml of a 2:95 ethanol: 1:2 N KOH solution three times for 10 min each in a boiling water bath. The resulting pellet was washed with distilled water three or four times or until the washings showed a neutral pH. Wall purity was determined by phase and electron microscopy and the decreasing level of protein found in the washings. The second method followed closely that described by Lin *et al.* (1976) with a 4 min sonication at 30 watts. Cleaned walls were dried by lyophilization (Virtis Research Equipment) and stored over desiccant until further use. All subsequent analyses were begun with 100 mg samples of this material. Total glucan was determined on each preparation of cleaned walls by the anthrone method (Morris 1948; Dische 1962).

Chemical Fractionation of the Wall

The cleaned and freeze-dried walls were chemically fractionated by three successive treatments. The first was acid (0.5 N HCl) (Aronson *et al.* 1967; Sietsma *et al.* 1969) and the second was alkali (2 N KOH). The third treatment used was either Schweitzer's reagent (personal

communication Dr. J. M. Aronson) or cadoxen (Jayme and Neuschaffer 1957; Jayme and Lang 1963), both known cellulose solvents. The acid-soluble fraction was obtained by five 30 min treatments at 70°C of 100 mg wall material in 50 ml 0.5 N HCl. The supernatants from each treatment were pooled and brought to a final concentration of 85% ethanol and allowed to stand overnight at 4°C. The ethanol precipitated polysaccharide was collected the next day by centrifugation, freeze-dried, and stored over desiccant until further use. The pellet which remained from the acid extraction was washed with distilled water until neutral, and then treated with 2 N KOH in the same manner as the acid treatment. This became the alkali-soluble fraction. The remaining pellet was dissolved in either one of the two cellulose solvents, with cadoxen being favored because of its colorless and odorless nature (Ladisch *et al.* 1978). The basic procedure for cellulose dissolution was the same with both solvents and both appeared equally effective. The pellet remaining from the acid-alkali extractions was treated overnight under N₂ with 40 ml of freshly prepared reagent at room temperature with stirring. Two additional 2 hr extractions were performed and finally the supernatants were pooled and treated with glacial acetic acid until neutral. The solution was centrifuged at 48 300 x *g* for 20 min in a Beckman J2-21 refrigerated centrifuge. The pellet was washed once with 1 N acetic acid, twice with distilled water, twice with 22% NH₄OH for the Schweitzer's reagent or 30% ethylenediamine for the cadoxen reagent (the first time for 30 min), once with 1 N acetic acid, and finally with distilled water. The pellet was freeze-dried and stored over desiccant for further study. This is the Schweitzer's or cadoxen reagent-soluble fraction, and is termed cellulose II (regenerated cellulose) (Preston 1974a). The

pellet remaining was neutralized by washing with distilled water and freeze-dried as above. This is the insoluble residuum.

Cellulose I or native cellulose and chitin were extracted from frozen mycelia or isolated walls following the method of Aronson and Lin (1978).

Chemical Analyses of Wall Constituents

Preparation of Material for Neutral Sugar Analyses

Enzyme hydrolysis

Lyophilized walls or their derived fractions were treated with various enzymes and the products of hydrolysis were determined. The enzymes used were laminarinase (E. C. 3.1.1.6, β 1,3-glucanase *ex mollusca*, B grade, CalBiochem), cellulase (E. C. 3.2.1.4, β 1,4-glycanohydrolase from *Aspergillus niger*, Type I, Sigma), chitinase (E. C. 3.2.1.14, chito-dextrinase, poly(1,4- β -[2-acet-amido-2-deoxy]-D-glucoside) glycanohydrolase from *Streptomyces griseus*, Sigma), protease (from *S. griseus*, Type VI, Sigma), and lipase (448 from hog pancreas, Nut. Biochem. Co.). The buffer used for cellulase and laminarinase was 0.05 M sodium citrate pH 5.0; for chitinase 0.05 M phosphate pH 6.0; for protease 0.05 M HEPES pH 7.6 or 0.05 M sodium phosphate-citrate pH 7.6; and for lipase 0.05 M sodium phosphate-citrate pH 6.2. Reactions were allowed to run 24 hr unless otherwise specified. The chitinase and lipase reactions were carried out at 25°C and the others at 37°C. The concentration of enzyme was 500 μ g/ml and that of the substrate 2 mg/ml. Bacterial contamination was prevented by the addition of 100 μ g/ml streptomycin, 500 μ g/ml merthiolate, or when gas chromatographic analyses were to follow, a toluene layer

covering the reaction mixture. Tests for enzyme purity were performed by reacting the enzyme in question with a known substrate. They were: laminarin,¹ from *Laminaria digitata*, A grade, anhydroglucose 94%, CalBiochem; cellulose, carboxymethylcellulose or acid swollen Whatman Ashless Powder for chromatography (Reese and Mandels 1963b; Green 1963); chitin, purified powder from crab, Sigma; Tween 20; and bovine albumin powder, Fr. V, 96-99%, Sigma. The conditions of enzyme hydrolysis are compiled in Table 1. Two of the enzymes were found to be active against more than one substrate. Laminarinase was found to be active against both laminarin and cellulose, and cellulase was active against both protein and cellulose (Whitaker 1970). Thus, it was necessary to purify these two enzymes before use (Sietsma *et al.* 1968).

Assays for enzyme activity

The activity of laminarinase was measured by determining the increase in total reducing sugar or in glucose over that of the enzyme or substrate alone, using the anthrone method (Morris 1948; Dische 1962) or the glucostat test (Worthington Biochemical Corp.). Cellulase activity was determined viscometrically (Thomas and Mullins 1969). The production of N-acetylglucosamine, as determined by the DMAB method (Reissig *et al.* 1955), was used to measure the chitinase activity. The activity of protease was determined by the decrease in total protein, as measured by the BioRad technique (BioRad Technical Bulletin #1051). The release of fatty acids, as determined by a change in pH, was used to measure the activity of lipase (Bier 1955).

¹This substrate contained β 1,3 linkages only. Another laminarin (source unknown) was also used which was found to contain β 1,6 linkages in addition to the β 1,3.

Table 1. Conditions of enzyme hydrolysis.

Enzyme	Purveyor	Concentration of Enzyme/ml	Concentration of Substrate/ml	Incubation Time	Temperature	Activity Under Spec- ified Condi- tions ^a
Cellulase E.C. 3.2.1.4 (<i>Aspergillus niger</i>)	Sigma	500 µg (60 u)	12 mg	24 hr	37°C	60 µ ^b 12 u
Cellulase (<i>Achyla ambisexualis</i>)	---	5 u	2.5 mg	168 hr	30°C	5 u ^c
Laminarinase E.C. 3.1.1.6 (ex mollusca)	CalBiochem	500 µg	2 mg	24 hr	37°C	500-600 µg ^d 48 µg ^e
Lipase (hog pancreas)	Nut. Biochem	500 µg	2 mg	24 hr	37°C	10.8 u
Protease (<i>Streptomyces griseus</i>)	Sigma	500 µg	2 mg	24 hr	37°C	1.93 mg
Chitinase E.C. 3.2.1.14 (<i>Streptomyces griseus</i>)	Sigma	500 µg	2 mg	336 hr	25°C	152 µg

^a per 500 µg enzyme where possible^b 120 units/mg unpurified enzyme, 12 units/ml purified enzyme; both per hr^c per ml enzyme per hr^d per 2 mg wall, determined by the anthrone reagent^e per 2 mg wall, determined by the glucostat assay^{d,e} both purified and unpurified enzymes have the same activity

Acid hydrolysis

Unfractionated walls and the wall fractions described previously were hydrolyzed in sealed ampules for varying periods of time with 6 N HCl under N_2 at room temperature. The resulting hydrolysates were then analyzed for mono- and disaccharides. Before analysis the hydrolysates were diluted with distilled water and either dried over NaOH pellets *in vacuo* until they were neutral or were deionized by passage through a Dowex carbonate column and then dried *in vacuo*. The sample size was 2 mg per 0.5 ml 6 N HCl. Unfractionated walls were treated for 48 hr, the acid- and alkali-soluble fractions and the insoluble residuum for 4 hr, and the cellulose II fraction for 96 hr. The anthrone reagent was used to determine total glucan in these samples.

Neutral Sugar Analyses

All neutral sugar analyses were performed on chemically cleaned walls or the fractions prepared from these walls.

Acid and enzyme hydrolysates of unfractionated walls and their fractions were analyzed by both paper and gas-liquid chromatography, the latter by the formation of trimethylsilyl derivatives (Sweeley *et al.* 1963; Zanetta 1972). Single dimension descending paper chromatography was done on Whatman #1 chromatography paper, 46 by 56 cm, in a solvent saturated Chromatocab (Research Specialties Co.) at room temperature for 24 hr following known procedures (Kowkabany 1954; Block *et al.* 1958). Material containing 100 μ g or more carbohydrate was applied to the paper. The solvent system used was 6 butanol: 4 pyridine: 3 water. Dried chromatograms were developed in a 105°C oven after having been sprayed

with aniline phthalate (Partridge 1944). R_g values were calculated from the resulting spots and compared with known standards.

Derivatization of samples and standards for GLC was done in the following manner. Solutions containing known amounts of carbohydrate were first lyophilized and then volatilized by a 10 min treatment with 0.2 ml dried pyridine, 0.1 ml hexamethyldisilazane and 0.1 ml trimethylchlorosilane when only monosaccharides were present, or for 3 hr when disaccharides were (Ishizuka *et al.* 1966; Bhatti *et al.* 1970). The reactants were mixed on a vortex and after gentle warming (Yamakawa and Ueta 1964a, b) the reactions were carried out at room temperature. At the end of the reaction period, the mixture was dried with a stream of N_2 in a warm water bath, and the derivatives were extracted with 0.4 ml methylene chloride (Mallinckrodt, nanograde). Sample size varied from 2 to 6 μ l depending on the amount of carbohydrate, and sample concentration varied from 10 to 20 μ g carbohydrate.

The gas chromatograph used was a Hewlett-Packard F & M 402 with dual flame ionization detectors. The carrier gas was helium. The columns were standard 1.8 m tubes with internal diameters of 3 mm. The packing material was 3% (w/w) JXR on 100-120 mesh Gas Chrom Q. Two different temperature programs were used. Monosaccharide separations used a starting temperature of 170°C for 5 min, followed by a rise of 2°/min to 210°C where the temperature was held. Disaccharide separations used a similar temperature regime, except that once 210° was reached the program was changed to a 10°/min increase to 240° where the temperature was held. The internal standard which was incorporated at the time of acid or enzyme hydrolysis was myo-inositol in the monosaccharide program

and sucrose in the disaccharide. Peak areas were determined by the use of a K & E Compensating Polar Planimeter (620 005) and the relative quantities of the disaccharide components found were calculated on the basis of the internal standards (Davison and Young 1969; Clamp *et al.* 1971).

Linkage and branching analyses of the various polysaccharides isolated from the wall were done by periodate oxidation. The following procedure was modified from that of several previous ones (Dyer 1956; Goldstein *et al.* 1965; Hay *et al.* 1965). A 36.7 mg sample was dissolved in 25 ml of 0.04 M sodium metaperiodate (Sigma), which had been dissolved in acidified water (pH 4.5), and placed in flasks which were covered with black electric tape and aluminum foil. Aliquots were taken immediately for T_0 and analyzed. The materials to be oxidized were placed on a wrist-arm shaker at 4°C and aliquots were removed for analysis every 24 hr for a total period of 120 hr. Periodate ion consumption was determined by UV absorption at 222.5 nm of 0.1 ml samples after a 250-fold dilution. Formic acid liberation was determined by titration with 0.01 N NaOH on 1 ml samples, to which 0.1 ml acid free ethylene glycol and, after 10 min at room temperature, 0.5 ml 0.02% methyl red had been added. The amount of base necessary for neutralization was then correlated with the amount of formic acid in the sample. Appropriate controls were also analyzed.

Live hyphae with developing branches plus samples of isolated cleaned walls and their fractions were observed under a polarizing light microscope. The pattern of birefringence was noted.

Samples of both Schweitzer's and cadoxen reagent-soluble material were subjected to x-ray diffraction analyses by Dr. J. M. Aronson of the

Department of Botany and Microbiology, Arizona State University, Tempe, Arizona.

Amino Sugar Analyses

Solubility of the hexosamine component of the wall

The procedure used was that of Lin *et al.* (1976). The three major fractions were first lyophilized and then hydrolyzed with 16 ml of 4 N HCl at 98°C under N₂ in sealed ampules. After a 16 hr reaction period, the ampules were opened and the acid was removed by rotoevaporation. The remaining contents were washed three times with distilled water and finally dissolved in 5 ml 0.01 N HCl for amino sugar analysis in an automated Amino Acid Analyzer (Model JLC-6AH, Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan).

Lugol's iodine detection of chitin

Lugol's iodine was prepared as a 1 iodine: 2 potassium iodide: 300 distilled water solution. The test material was placed in a depression slide in a few drops of oxalate buffer ranging in pH from 1.6 to 4.0. A few drops of the Lugol's iodine was added and the material was observed under a light microscope to determine any color development (Prakasam and Azariah 1975).

Uronic Acid Analysis

The procedure for isolating uronic acids follows that of Gancedo *et al.* (1966). The presence and quantity of uronic acid was determined by the carbazole test (Bitter and Muir 1962).

Protein and Amino Acid Analyses

Total protein was determined on wall samples which had been washed in buffer and water. They were then homogenized with a glass tissue grinder (Kontes Glass Co.) in 1 N NaOH and the resulting homogenate was placed at 50°C for 3 hr. Protein was determined by the BioRad method (BioRad Technical Bulletin #1051 1977).

Amino acid profiles were determined on 200 and 20 mg samples of chemically cleaned and of buffer-water washed walls, respectively, following hydrolysis in 6 N HCl at 105°C. Similar profiles were also determined on 20 mg samples of walls taken at various stages during chemical cleaning. These stages were: (1) supernatant after initial pelleting subsequent to grinding; (2) buffer washed once; (3) buffer washed once plus sonicated and heated 30 min at 90°C in 2% SLS; and (4) walls from (3) treated once 10 min in boiling water with ethanolic KOH. After treatment for 48 hr the acid was removed by rotoevaporation as described previously and the amino acids were analyzed by an automated Amino Acid Analyzer.

Lipid Analysis

The procedure of extracting readily extractable lipids was that of Kanfer and Kennedy (1963). The dried extract was spotted on Silica Gel G plates activated with iodine and detection was by double bond formation with iodine (Whitehouse *et al.* 1958). The solvent used for ascending chromatography was 65 chloroform: 25 methanol: 8 glacial acetic acid (Ames 1968). The pattern of spots suggested phospholipid. A Fiske-Subbarow solution (Bartlett 1959) plus 0.5 ml 10 N H₂SO₄ was sprayed onto the dried plates to detect phosphorus.

Phosphorus Analysis

Total phosphorus was determined on wall samples which had been combusted at 160°C in 10 N H₂SO₄ and H₂O₂ for 48 hr. Phosphorus was measured by the Fiske-Subbarow method (Barlett 1959).

Hydrolysis of Buffer-Water Washed Walls by *A. ambisexualis* Cellulase

A sample of the enzyme cellulase was extracted with acetone from medium in which *Achlya* had grown on the enriched formula (Kane 1971) for 48 hr. Enzyme precipitation was achieved by adding 2 volumes of acetone to the medium, followed by centrifugation at 12 100 x *g* for 20 min. The pellet was then resuspended in distilled water at a ratio of 1 ml per gm fresh weight of mycelium, and centrifuged at 18 800 x *g* for 15 min. The supernatant was dialyzed for 24 hr against a 0.018 M citrate-NaOH buffer pH 5.0 with 0.05% merthiolate to remove glucose present in the original medium. Viscometric assay of this enzyme solution revealed an activity of 5 units/ml (Thomas and Mullins 1969). Isolated buffer-water washed walls were prepared as usual except that they were not lyophilized, and a final washing with the above citrate-NaOH buffer was made. One ml of the enzyme solution and 2.5 ml of the wall suspension were added to a 15 ml conical centrifuge tube and placed at 30°C for 168 hr. Aliquots of 0.2 ml were taken at 24 hr intervals and the total reducing sugar was measured in the supernatant by the anthrone method (Morris 1948; Dische 1962).

Ultrastructural Studies

Surface structure of live hyphae, isolated walls, and wall fractions was studied under a number of varying regimes of chemical and enzymatic treatments.

Surface Structure of Chemically Treated Walls

Isolated walls were first treated with 0.5 N HCl and placed in a 70°C water bath for 30 min. This treatment was repeated five times. Surface replicas were made of samples of the wall which remained. The rest of the remaining wall was treated similarly but with 2 N KOH. Samples of the wall left from this treatment were taken for surface replication. The residual wall material was treated with cadoxen reagent and surface replicas of the insoluble material were made. The cadoxen-soluble material was treated with acid to regenerate cellulose II and surface replicas were again made of this component.

Surface Structure of Wall Fractions

Surface replicas of each of the wall fractions described in the section on Chemical Fractionation of the Wall were made.

Surface Structure of Chemically Treated Live Hyphae

Live hyphae were treated in the same manner as the isolated walls and replicas were made of the wall surfaces of the hyphal samples after each treatment. Replicas were not made, however, of material which was solubilized in cadoxen.

Surface Structure of Enzymatically Treated Live Hyphae

Very small amounts of 48 hr old mycelium were placed in the wells of a plastic Tissue Culture Cluster Chamber (Costar) and 0.2 ml of the various enzyme solutions containing merthiolate were added. Sterile cotton, soaked in sterile water, was placed in nearby wells to prevent desiccation. The reaction mixtures were placed at 37°C for 48 hr. At the end of the incubation time hyphae were removed from the well, washed with sterile water, and placed on freshly cleaved mica for drying and eventual surface replication. Hyphae, which were to be treated with a second enzyme, were washed and returned to the well and the second enzyme was added. If a third enzyme was to be added, the same procedure was repeated. Assay conditions were the same as those described in the section on enzyme hydrolysis. The activities of the enzymes used were: laminarinase (purified), 620 µg reducing sugar (as glucose) released from 2 mg cell wall/ml enzyme; *Aspergillus niger* cellulase (purified), 12 units/ml; *Achlya ambisexualis* cellulase, 5 units/ml; and protease, 500 µg/ml. Controls for each sample contained boiled enzyme.

Diameter of Microfibrils

The width of microfibrils was determined from negatives of carbon-platinum surface replicas taken at 33 K and 50 K magnification under varying preparative conditions. These conditions were treatment with buffer (untreated), laminarinase, laminarinase-protease, and 0.5 N HCl followed by 2 N KOH. The diameter of cellulose I microfibrils was also determined.

Preparation of Replicas

Single-stage carbon-platinum replicas (Pease 1964; Bradley 1965) were made of the wall surface by the following procedure. Samples of the treated wall were air-dried on mica and were shadowed with platinum at an angle of 45° in a Balzer's High Vacuum Coating Unit Micro-BA 3 or a Balzer's BA 360 Freeze Etch Device. After shadowing, the specimens were coated with carbon. Biological material and the mica were removed from the replicas by floating on 40% chromic acid solution. The replicas were washed twice with distilled water and allowed to sit overnight in 50% chlorox. The chlorox was washed off by two 15 min washes with distilled water. The replicas were placed on 100 mesh copper formvar coated grids and were examined by a Hitachi HU-11E or a Jeolco JEM-100 Cx electron microscope.

RESULTS

Criteria for Wall Purity

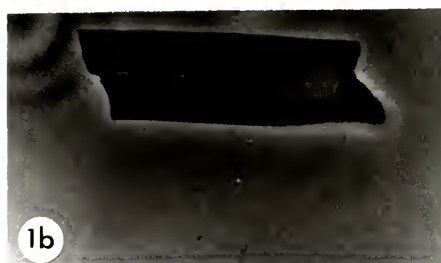
Observations of both chemically cleaned and buffer-water washed walls with phase and electron microscopy revealed that they were relatively free of cytoplasmic contaminants (Fig. 1 a and b). In addition, there was a decrease in the protein content of the wall washings during the successive stages in the chemical cleaning process as shown in Fig. 2.

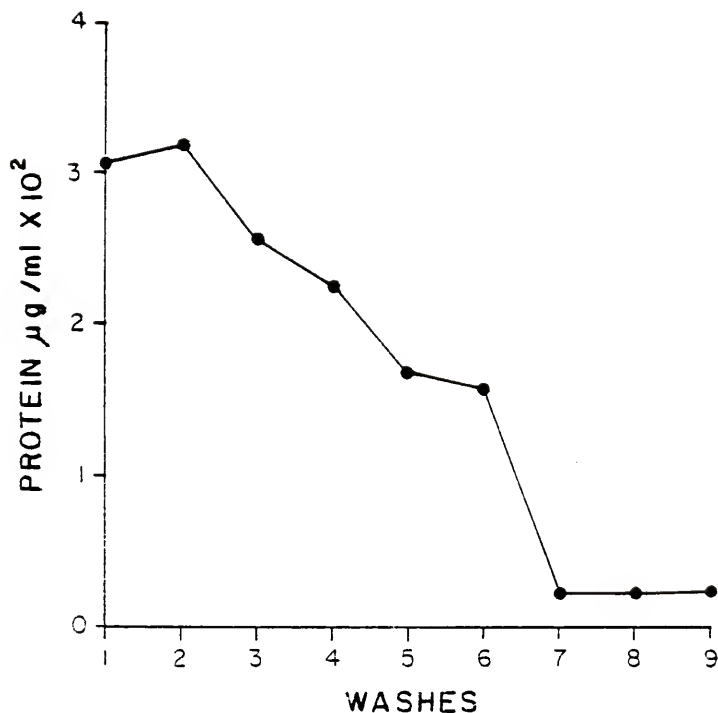
The two methods of wall cleaning gave quite different amounts of dried cleaned walls per original gm fresh weight of mycelium. About twice as much wall material was obtained after buffer-water washing as after chemical cleaning, 6.82 mg and 3.20 mg dried walls/gm fresh weight of mycelium. Total glucan content of both preparations, as measured by the anthrone method, was quite similar: 52.80 mg and 56.00 mg/100 mg dried walls for buffer-water washed and chemically cleaned, respectively.

Chemical Fractionation of the Wall

Table 2 gives the results of the chemical fractionation of the carbohydrate component of the wall. That portion of the wall soluble in weak acid was 37.58%, while only 7.07% of the remaining wall was soluble in alkali. Some 20.83% was soluble in cellulose solvents, leaving a 5.58% residuum. These four fractions account for 71.06% of the wall and were shown to consist of the following: (1) acid-soluble = β 1,3

Figure 1. Phase contrast photographs of cleaned walls.
(a) Chemical. X 800. (b) Buffer-water. X 800.





1. Buffer suspension after grinding
2. Detergent treated, sonicated, and heated
3. 2 ethanol: 1 KOH
4. 2 ethanol: 1 KOH, 10 min boiling, 1X
5. 2 ethanol: 1 KOH, 10 min boiling, 2X
6. 2 ethanol: 1 KOH, 10 min boiling, 3X
7. Water, 1X
8. Water, 2X
9. Water, 3X

Figure 2. Decrease in total protein^a as a measure of wall purity.

^adetermined by the Lowry method

Table 2. Carbohydrate fractions of *A. ambisexualis* wall.

Fraction	mg ^a
Acid-soluble	37.58
Alkali-soluble	7.07
Cellulose II	20.83
Insoluble residuum	5.58
Cellulose I	19.06
Chitin	0.63
Total glucan ^b	52.8 ^c , 56.0 ^d

^a average of three determinations based on 100 mg samples

^b determined (as glucose) with anthrone on unhydrolyzed walls; calculated as anhydroglucose

^c buffer-water washed walls

^d chemically cleaned walls

and β 1,6 glucan; (2) alkali-soluble = β 1,3; β 1,4 and β 1,6 glucan; (3) cellulose II = β 1,4 glucan; and (4) insoluble residuum = β 1,3; β 1,4 and β 1,6 glucan.

The amount of cellulose I was 19.06% of the wall and this is 2% less than the value for cellulose II. A very small portion of chitin was found (0.63%) and some additional tests were made to support this identification. A two week incubation of this component with chitinase released about the same level of N-acetylglucosamine as did a known chitin substrate. A 700 μ g sample of wall material released 58 μ g of N-acetylglucosamine and a 2000 μ g sample of crab chitin released 152 μ g. A spot identified as N-acetylglucosamine was obtained with paper chromatography from both enzyme hydrolysates (Table 3). Cytological staining with Lugol's iodine compared favorably with known samples of chitosan and deacetylated chitin.

Chemical Analyses of Wall Constituents

Neutral Sugars

The above fractions (Table 2) were then analyzed for their monosaccharide composition, types of glycosidic linkage, and pattern of branching. The acid-soluble fraction was hydrolyzed and the products separated by paper chromatography. They consisted of the monosaccharide glucose and the disaccharides laminaribiose and gentiobiose (Table 4). Positive identification of a probable trisaccharide spot was not possible because of conflicting R_g values between laminaritriose and cellotriose. The two disaccharides found indicated the presence of two different linkages, β 1,3 and β 1,6, respectively. The alkali-soluble fraction

Table 3. The separation of enzyme hydrolysates of wall fractions of *A. ambisexualis* by paper chromatography.

Wall Fraction + Enzyme	P r o d u c t s					
	glucose	laminaribiose	cellobiose	gentiobiose	trisaccharide	N-acetylglucosamine
Acid-soluble cellulase ^a laminarinase	+	- +	- -	- +	- +	- -
Alkali-soluble laminarinase	+	+	-	-	-	-
Cellulose II cellulase laminarinase	+	- -	- -	- -	- -	- -
Insoluble residuum laminarinase	+	+	+	-	-	-
Total wall cellulase laminarinase	+	- +	- -	- -	- -	- -
Chitin chitinase	-	-	-	-	-	+

^a from *Aspergillus niger*

Table 4. The separation of acid hydrolysates of wall fractions of *A. ambisexualis* by paper chromatography.

Wall Fraction	P r o d u c t s				
	glucose	laminaribiose	cellobiose	gentiobiose	trisaccharide
Acid-soluble	+	+	-	+	+
Alkali-soluble	+	+	+	^a +	+
Cellulose II	+	-	+	-	+
Insoluble residuum	+	+	+	^a +	+
Cellulose I	+	-	+	-	-
Total Wall	+	-	+	+	+

^a very light spot seen

revealed glucose, laminaribiose, gentiobiose, and cellobiose. Both the cellulose I and II fractions gave glucose and cellobiose with the same analysis. The residuum, remaining after removal of the three above fractions, produced small amounts of glucose, laminaribiose, gentiobiose, and cellobiose upon hydrolysis. The unfractionated wall produced all of the above sugars except laminaribiose, even after a short (2 hr) hydrolysis period. Both the unfractionated wall and cellulose II contained a compound which was unidentifiable by R_g value.

Before presenting the results of the enzyme hydrolysis studies, some comments on the properties of the purified enzymes will be made. The purified laminarinase (see Materials and Methods) when reacted with a laminarin containing only $\beta 1,3$ linkages, produced glucose, laminaribiose, and laminaritriose. When, however, it was presented with a laminarin containing mixed linkages of $\beta 1,3$ and $\beta 1,6$, gentiobiose appeared with the above products. This enzyme preparation thus behaves like both an $\text{exo-}\beta 1,3$ -glucanase and an $\text{endo-}\beta 1,3$ -glucanase. The purified cellulase preparation obtained from *Aspergillus niger* exhibited activity against carboxymethylcellulose in the viscometric assay and is thus classified as an endo- or random splitting enzyme (Reese and Mandels 1963a). When this enzyme was reacted with wall fractions or the intact wall no chromatographable compounds were formed in the reaction time used. However, treatment of the wall or its fraction with unpurified enzyme yielded glucose. The unpurified enzyme preparation also is able to hydrolyze sucrose, as this disaccharide was never obtained in GLC analysis, even though it was added as an internal standard at the time of hydrolysis (Fig. 3e).

Figure 3. GLC of the TMS derivatives of the monosaccharides released by hydrolysis of the wall fractions or the total wall by unpurified *A. niger* cellulase. (a) Acid-soluble. Glucose is the only product. (b) Boiled enzyme control. Small peaks of mannose and glucose are contaminants of the enzyme and substrate preparations respectively. (c) Cellulose II. Glucose is the only product. (d) Boiled enzyme control. (e) Total wall. Glucose is the only sugar produced. The fructose and some of the glucose result from the action of β -glucosidase, present as a contaminant in the enzyme preparation, on sucrose added as the internal disaccharide standard. (f) Boiled enzyme control.

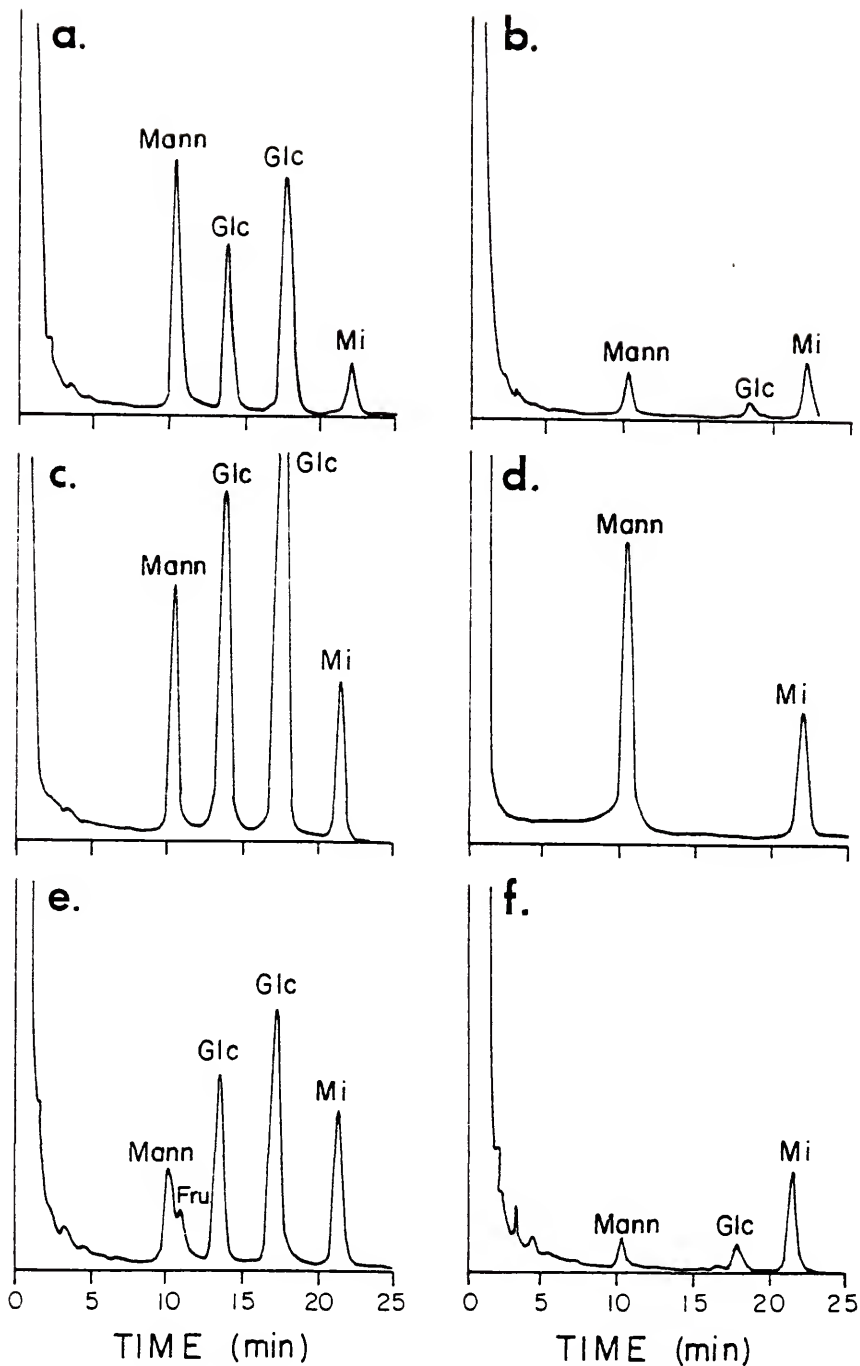
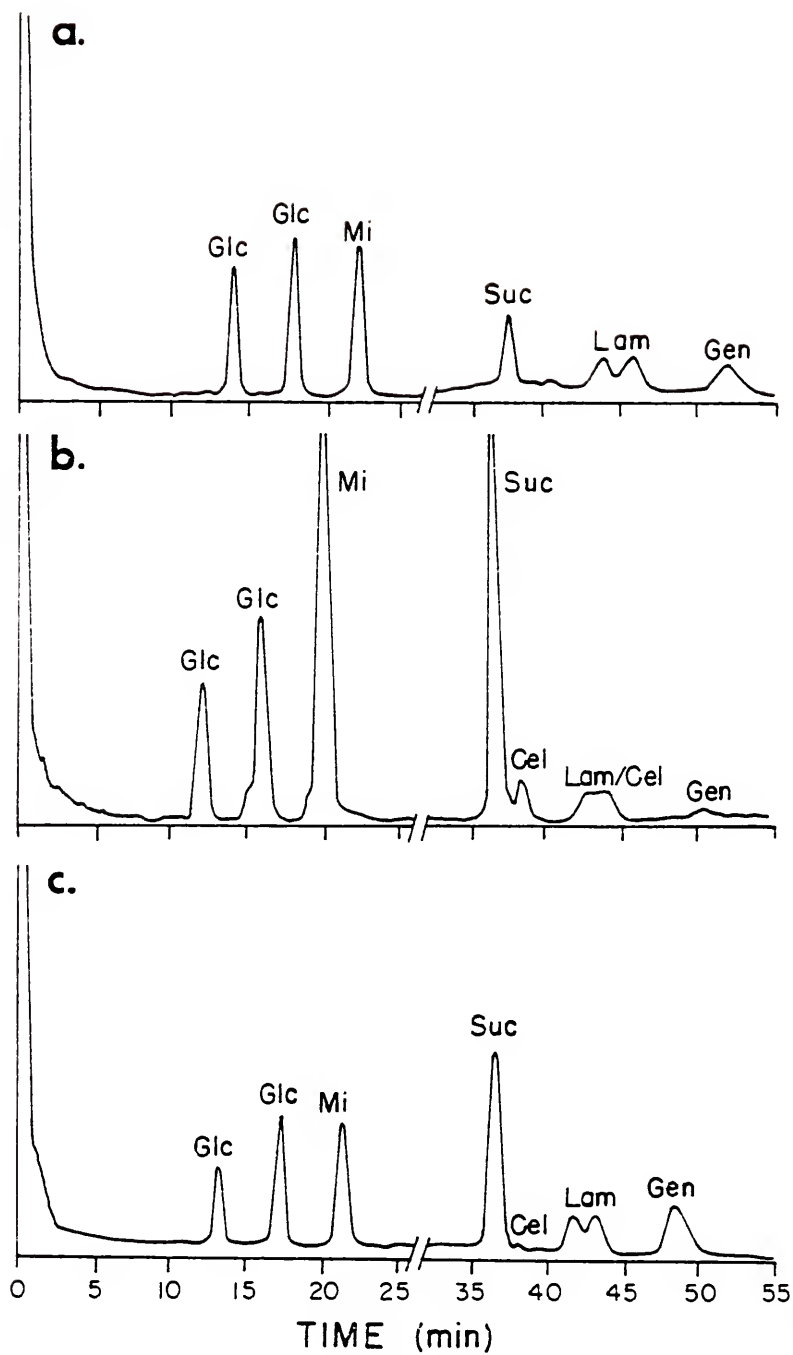


Table 3 gives the results of enzyme hydrolysis of the various wall fractions. Neither lipase nor protease showed activity against any of the wall fractions. Purified laminarinase hydrolyzed the acid-soluble portion of the wall releasing glucose, laminaribiose, and gentiobiose. Glucose was released from all the fractions, except the chitinous one. Laminaribiose was found in the unfractionated wall after treatment with laminarinase. This disaccharide was also found in laminarinase hydrolysates of the alkali-soluble fraction and the insoluble residuum. Based on the R_g value, cellobiose was also found with laminarinase treatment of this residuum.

In a number of cases an unidentified spot appeared with a variable R_g value. It was always higher than laminaribiose but lower than glucose. The R_g value of this spot was a little higher than that found in some of the acid hydrolysates. All the control enzyme and substrate solutions were chromatographed, and no spots were found. From the GLC studies, a compound which cochromatographed with mannose, was found associated with both laminarinase and cellulase; however, the R_g value of authentic mannose is higher than glucose for this solvent system.

The data obtained from the GLC studies is similar to that found with paper chromatography. Acid hydrolysates (Fig. 4a-c) of the acid-soluble and cellulose II fractions plus the unfractionated wall yielded a mono- and disaccharide pattern similar to that found in the paper chromatographs. Some differences, however, were found. The hydrolysate of cellulose II consisted of not only glucose and cellobiose, but also small amounts of laminaribiose and gentiobiose (Fig. 4b). The gentiobiose component of this fraction was small, but it was difficult

Figure 4. GLC of the TMS derivatives of the mono- and disaccharides released by acid hydrolysis of wall fractions and total wall. (a) Acid-soluble. Products are glucose, laminaribiose and gentiobiose. (b) Cellulose II. Products are glucose, cellobiose, laminaribiose, and gentiobiose. (c) Total wall. Products are the same as in (b).



to determine the amount of the laminaribiose portion because there was an overlap between the two laminaribiose peaks and the second of the cellobiose. Acid treatment of the unfractionated wall revealed a pattern similar to that of the acid-soluble fraction, except for the presence of a small cellobiose peak (Fig. 4c). Although laminaribiose was not found in the paper chromatographic analysis of this hydrolysate, it was found in the GLC analysis.

Laminarinase treatment of fractionated and unfractionated walls is presented in Fig. 5a-f. Products from the enzymatic hydrolysis of the total wall and the acid-soluble fraction were glucose, laminaribiose, and gentiobiose (Fig. 5a and e). No products were obtained from laminarinase hydrolysis of cellulose II. Control samples containing boiled enzyme were also analyzed, and the results indicated that this treatment produced inactivation (Fig. 5b, d and f). The only substrate which had any residual sugar was the acid-soluble fraction which gave a very small glucose peak upon analysis. Treatment of the various fractions with cellulase gave results which were similar to the paper chromatographic studies (Fig. 3a-f). Glucose was the primary product found in these hydrolyses, and for this reason only the monosaccharide portions of these chromatographs are included.

Both laminarinase and cellulase were reacted with the unfractionated wall and only glucose was obtained (Fig. 6a and b). For some reason the mannose component was absent.

Since glucose was the only monosaccharide found, this datum was not quantified. The disaccharide components were quantitated based on their peak areas and that of a known standard (sucrose). The molar relative response factors for each of the components found were not calculated

Figure 5. GLC of the TMS derivatives of the mono- and disaccharides released by hydrolysis of the wall fractions or the total wall by laminarinase. (a) Acid-soluble. Products are glucose, laminaribiose, and gentiobiose. (b) Boiled enzyme control. Mannose is a contaminant of the enzyme preparation. (c) Cellulose II. (d) Boiled enzyme control. (e) Total wall. Products are glucose, laminaribiose, and gentiobiose. (f) Boiled enzyme control.

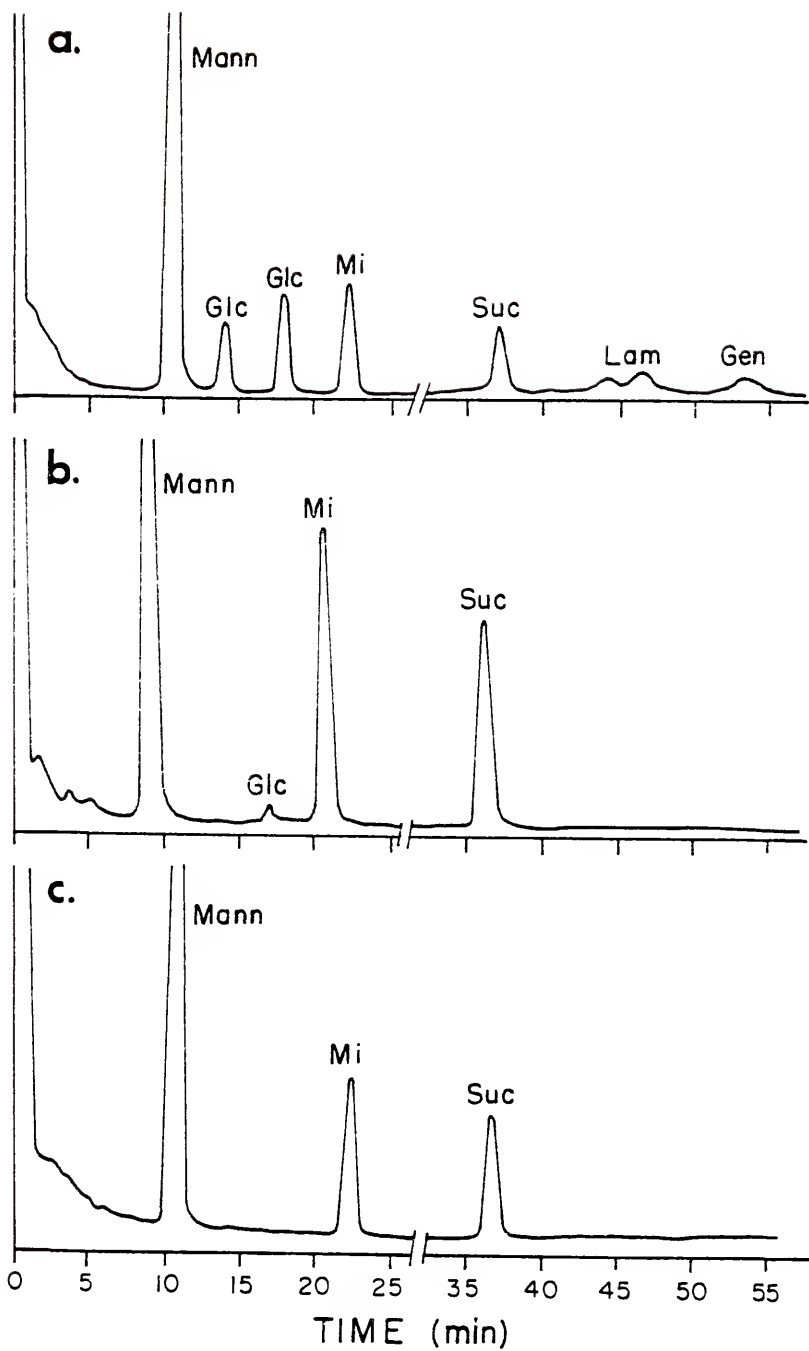
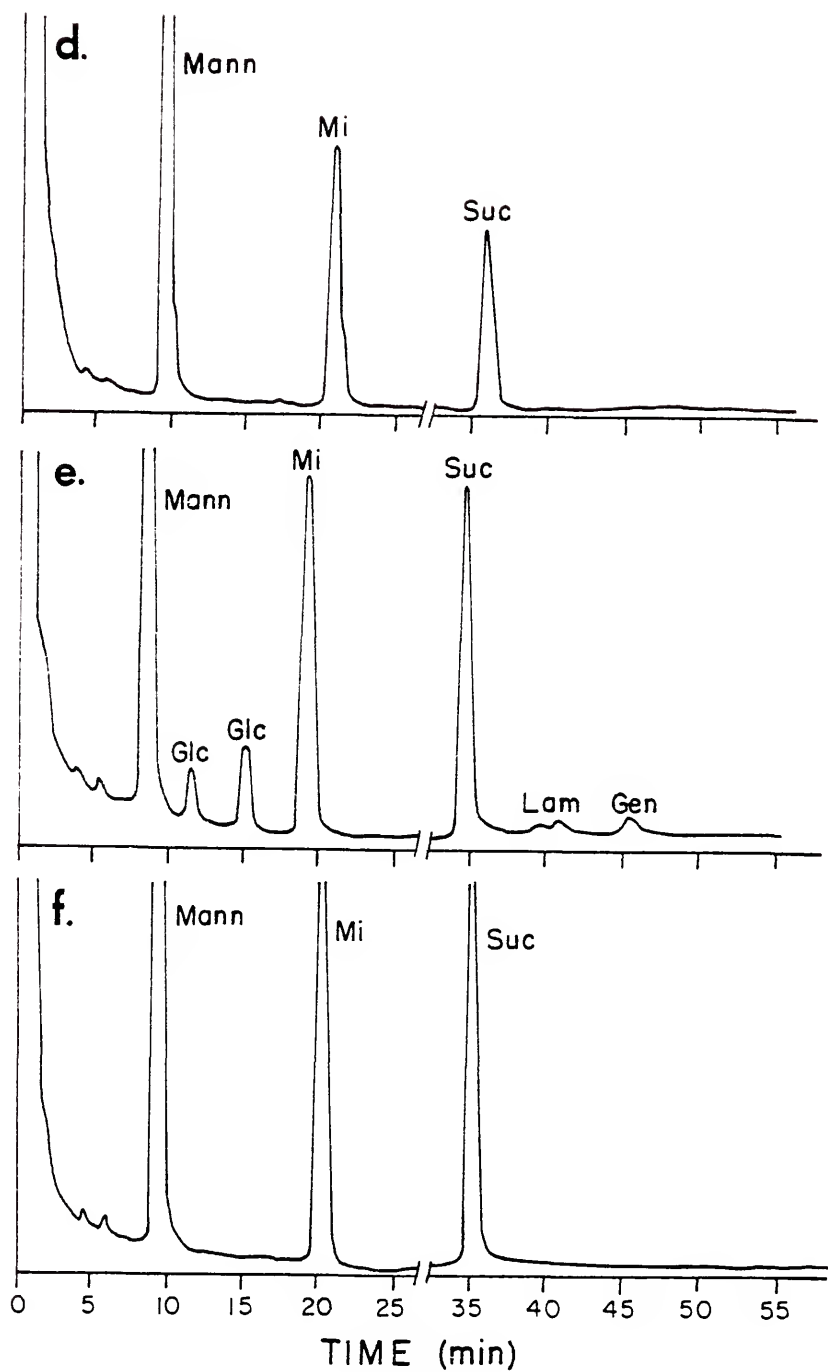


Figure 5. Continued.



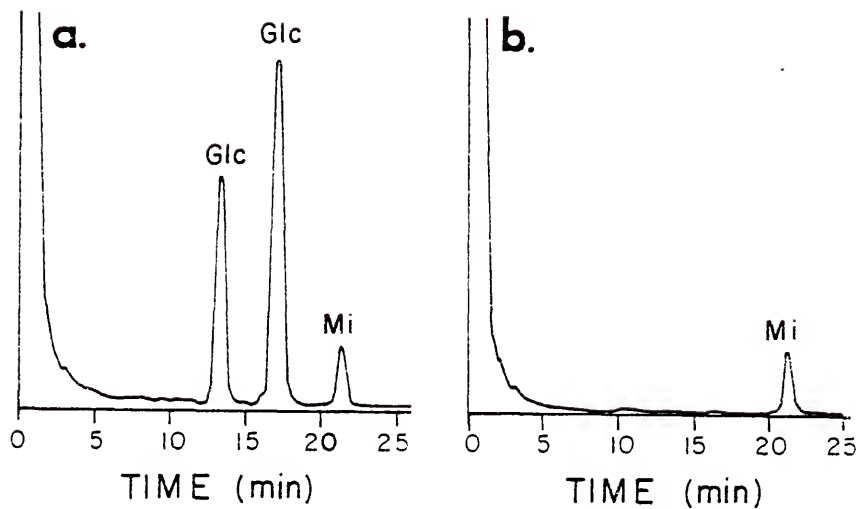


Figure 6. GLC of the TMS derivatives of the monosaccharides released by hydrolysis of the total wall with laminarinase and unpurified *A. niger* cellulase. (a) Total wall. (b) Boiled enzyme control.

because of variabilities in the system observed from day to day. It was felt that greater accuracy was obtained by comparing the areas of the peaks in question to known standards run simultaneously. The ratio of laminaribiose to gentiobiose in the acid-soluble fraction following acid hydrolysis was 1:0.72 and following enzyme hydrolysis was 1:0.57. In the unfractionated wall acid hydrolysis gave 1:1.02 and enzyme hydrolysis 1:0.9.

Periodate oxidation studies were done in order to gain some knowledge of the linkage and branching patterns in the various wall fractions. In addition to the studies of the wall fractions, three standard polysaccharides of known linkage patterns were also analyzed. These were cellulose powder (Whatman Ashless Powder, Chromatographic Grade) and the two different laminarins described previously. Table 5 gives the values of periodate consumption and formate liberation for the various wall fractions, and Table 6 for the polysaccharide standards.

Observations of unfractionated walls, wall fractions, and live hyphae under polarizing light revealed strong birefringence in the cellulose II fraction but none in the acid-soluble. Both live hyphae and isolated walls showed birefringence.

The results of x-ray diffraction analysis of cellulose II isolated by dissolution with Schweitzer's reagent or with cadoxen are presented in Table 7 and Fig. 7a and b. The lattice spacings of both preparations were the same as those found for the avicel cellulose II standard.

Amino Sugars

Because of the relatively large amount of glucosamine found in the samples analyzed for amino acids, a more detailed study of this

Table 5. Periodate consumption and formate liberation of *A. ambisexualis* wall fractions.

Fraction	Periodate ^{a,b}	Formate ^{a,b}
Acid-soluble	0.917	0.427
Alkali-soluble	0.609	0.091
Cellulose II	0.329	0.031
Insoluble residuum	0.610 ^c	0.031 ^c
Total Wall	0.963	0.213
Cellulose I	0.366	0.061

^a moles per mole glucose

^b values after 96 hr of treatment

^c values after 48 hr of treatment

Table 6. Periodate consumption and formate liberation of known polysaccharides.

Polysaccharide	Periodate ^{a,b}	Formate ^{a,b}
Whatman cellulose powder	0.370	0.031
Laminarin (β 1,3 linked)	0.159	0.152
Laminarin (β 1,6; β 1,3 linked)	0.329	0.152

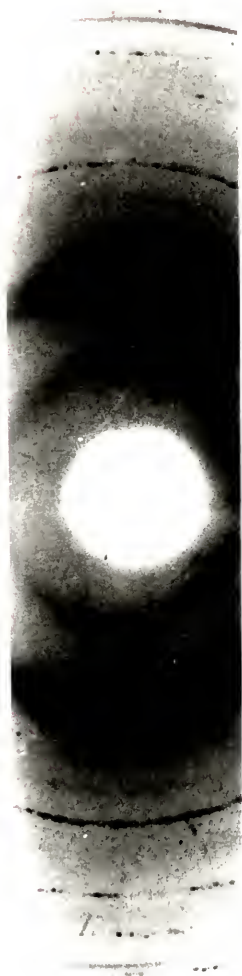
^a moles per mole glucose

^b values after 96 hr of treatment

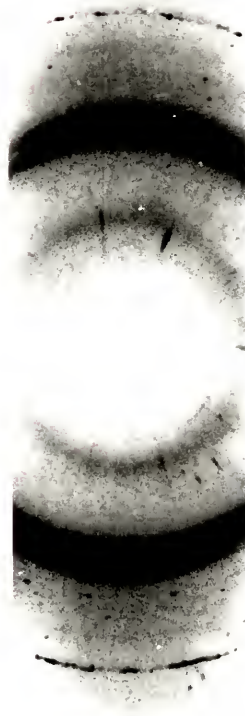
Table 7. X-ray diffraction analysis of Schweitzer's and cadoxen reagent-soluble fractions of *A. ambisexualis* wall.

Samples	Lattice spacings in Å		
Avicel standard	7.37	4.46	4.08
Schweitzer's	7.37	4.46	4.08
Cadoxen	7.37	4.46	4.08

Figure 7. X-ray diffraction patterns of cellulose II isolated from *A. ambisexualis* walls. (a) Cellulose dissolved with Schweitzer's reagent. (b) Cellulose dissolved with cadoxen.



7a



7b

monosaccharide was done. The solubility characteristics of the glucosamine component of the wall were studied and the results are given in Table 8. A large portion, 98.5%, of the glucosamine was insoluble in both dilute acid and base. The preliminary characterization of a small chitinous component isolated from the wall has already been described.

Uronic Acids

Preliminary studies of the uronic acid content of the wall gave 0.03 mg/100 mg dried wall prepared by buffer-water washing. No attempt was made to identify which uronic acids were present.

Protein and Amino Acids

Total protein of the untreated wall was 6 mg/100 mg dried wall after preparation by buffer-water washing. If, however, these walls were washed with 1 N NaOH and placed in a 50°C water bath for 3 hr, the total protein value increased to 10 mg/100 mg wall. Total protein was also measured in the various wall fractions. The acid-soluble fraction was the only one which showed the presence of measurable protein, 1.5 mg/100 mg dried wall. Traces of protein were found in the cellulose II fraction and in the insoluble residuum. Much of the protein was probably destroyed or washed away during the fractionation processes.

Amino acid analyses performed on both types of wall preparations showed distinct differences (Table 9). The chemically cleaned walls contained very low levels of amino acids and in some cases certain expected ones were missing, even when as much as a 200 mg wall sample was used. Walls which were buffer-water washed contained the whole spectrum of amino acids, including hydroxyproline (3%)(Table 9). A

Table 8. Analysis of solubility of glucosamine from unfractionated walls of *A. ambisexualis*.

Treatment	mg Glucosamine ^a
1 N NH ₄ OH, 25°C	0.039
1 N acetic acid, 98°C	0.000
Insoluble	<u>2.587</u>
Total glucosamine	2.626

^amg/100 mg dried wall, buffer-water washed

Table 9. Amino acid profile of the total wall of *A. ambisexualis* after chemical or buffer-water cleaning.

Amino Acid or Amino Sugar ^a	Chemical ^b	Buffer-water ^c
Glucosamine (average)	0.513	1.224
Lysine	0.009	0.298
Histidine	0.016	0.085
NH ₃	0.629	0.205
Arginine	0.015	0.207
Hydroxyproline	-----	0.103
Aspartate	0.009	0.187
Threonine	0.002	0.308
Serine	0.002	0.222
Glutamate	0.007	0.419
Proline	T	0.162
Glycine	0.007	0.166
Alanine	0.058	0.226
Cysteine	-----	0.043
Valine	0.006	0.206
Methionine	0.004	0.045
Isoleucine	0.004	0.172
Leucine	0.009	0.296
Tryosine	-----	0.104
Phenylalanine	<u>0.008</u>	<u>0.168</u>
Total amino acid	0.156	3.417

^a mg/100 mg dried wall

^b 200 mg sample used for hydrolysis

^c 20 mg sample used for hydrolysis

comparison of the total amounts of amino acids/100 mg dried wall revealed that the buffer-water washed walls contained nearly 22 times more amino acids than the chemically cleaned walls. The level of total protein in the buffer-water washed walls was much higher than the total amino acid content (10 mg and 3.42 mg respectively).

Examination of Table 10 and Fig. 8 indicates that the amino acid profile of the wall changed drastically during the chemical cleaning process. Detergent treated walls which were washed once with ethanolic: KOH followed by a 10 min incubation in a boiling water bath had a greatly reduced amino acid content. In a 20 mg sample only measurable amounts of lysine and methionine were present, and only trace amounts of aspartate, threonine, serine, glutamate, glycine, alanine, isoleucine, and leucine. Even detergent treated walls, which theoretically contained all covalently bound amino acids but not others, had a generally low level. These walls were also missing hydroxyproline, an amino acid generally found associated with cellulosic walls. The buffer washed walls (one washing) did not appear clean and most likely contained a lot of contaminating membrane proteins. The level of hydroxyproline was very low in these walls. Observations of Fig. 8 indicate that the profile in the buffer-water washed walls (washed until clean microscopically) in general follows that of the single buffer washing. The amino acid content of the supernatant after the initial pelleting represented only those amino acids which were soluble, as this fraction was not treated with any solubilizing agent. Significantly, hydroxyproline was missing from this sample. Strangely, histidine was also missing, and proline and cyteine were present in only trace amounts. In addition, glucosamine was not found.

Table 10. Comparison of amino acid profiles of samples of the total wall of *A. ambisexualis* during various stages of chemical cleaning.

Amino Acid or Amino Sugar	Supernatant After Initial Pelletings ^a	Buffer Washed One Time ^b	Buffer Washed One Time, Detergent Treated ^b	Ethanol- KOH ^b Treated
Glucosamine (average)	-----	1.120	0.780	0.990
Lysine	0.236	0.796	0.123	0.006
Histidine	-----	0.263	0.029	-----
NH ₃	0.089	0.236	0.060	0.032
Arginine	0.152	0.624	0.065	-----
Hydroxyproline	-----	T	-----	-----
Aspartate	0.452	1.117	0.180	T
Threonine	0.211	0.639	0.142	T
Serine	0.176	0.566	0.089	T
Glutamate	0.505	1.205	0.180	T
Proline	T	0.444	T	-----
Glycine	0.206	0.509	0.080	T
Alanine	0.351	0.746	0.120	T
Cysteine	T	0.100	T	-----
Valine	0.207	0.577	0.088	-----
Methionine	0.257	0.300	0.074	0.033
Isoleucine	0.172	0.454	0.073	T
Leucine	0.290	0.903	0.125	T
Tyrosine	0.083	0.445	T	-----
Phenylalanine	0.139	0.513	0.065	-----
Total amino acid	3.44	10.20	1.43	0.04

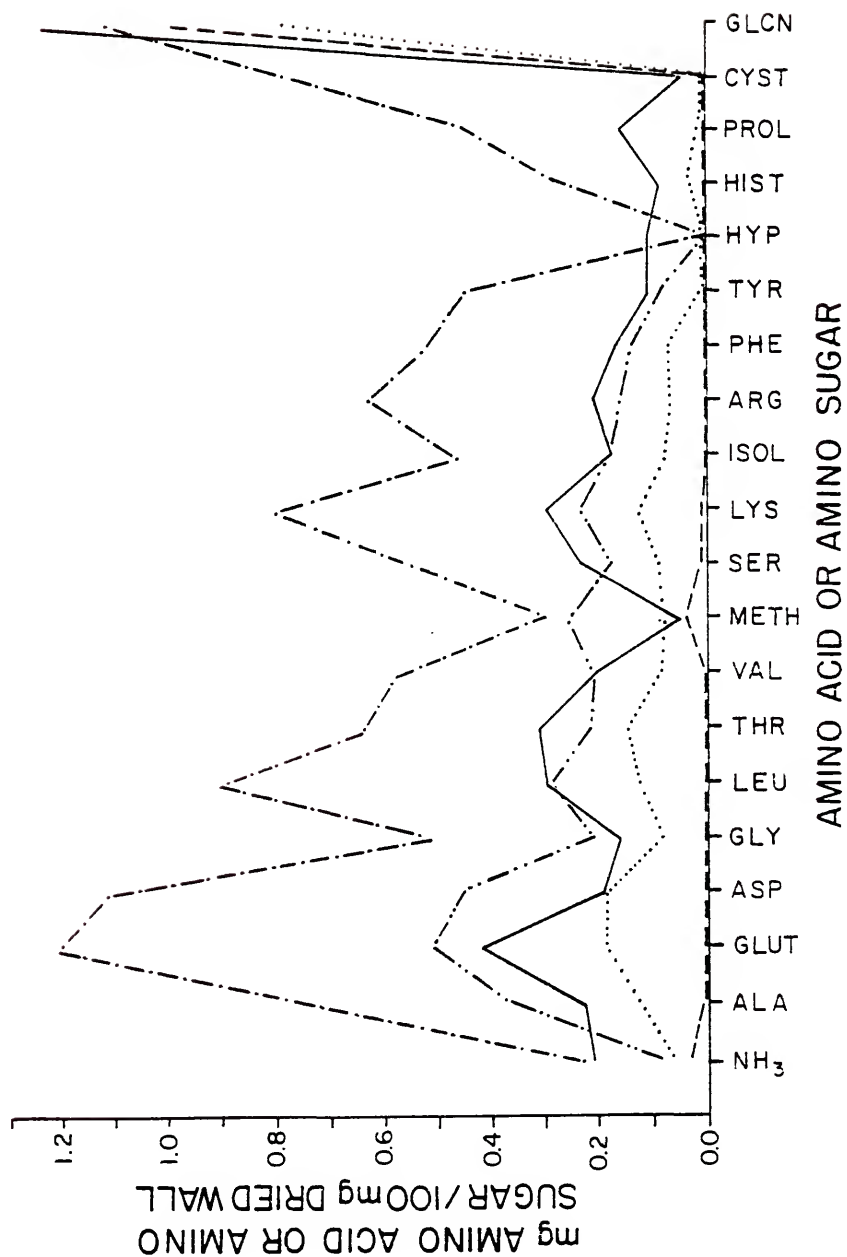
^a mg/100 mg sample

^b mg/100 mg dried wall

Figure 8. Comparison of amino acid profiles of samples of the total wall of *A. ambisexualis* during various stages of chemical cleaning. (---.---.--- supernatant after initial pelleting^a; ---.--- buffer washed, one time^a; . . . buffer washed one time, detergent treated^a; --- --- ethanollic-KOH treated^a; --- --- buffer-water washed until clean^b.)

^a 6N HCl, 12 hr, 105°

^b 6N HCl, 48 hr, 105°



Lipid

Only trace amounts of extractable lipids were found by the method used. The pattern of spots seen on thin layer chromatography plates suggested mainly phospholipids, and this was confirmed by a colorimetric spray test specific for these compounds.

Phosphorus

The level of phosphorus per 100 mg dried wall was 0.15 mg.

Total Wall Composition

The composition of the total wall is given in Table 11. When all the components were added the total represented 86.44% of the wall. The remaining 13.56% represented unidentified constituents or experimental losses.

Hydrolysis of Buffer-Water Washed Walls by *A. ambisexualis* Cellulase

The results of the hydrolysis of isolated walls by *A. ambisexualis* cellulase can be seen in Figs. 9 and 10. In the first 24 hr of hydrolysis, there was a significant increase in total reducing sugars in the treated wall samples as compared with the controls. This increase continued for another 72 to 96 hr, but at a reduced rate, and ceased between 96 and 120 hr.

Ultrastructural Studies

Surface Structure of Chemically Treated Walls

The replica of an untreated wall reveals a smooth surface (Fig. 11a). Walls treated with 0.5 N HCl appear somewhat less smooth than the

Table 11. Chemical constituents of the buffer-water washed walls of *A. ambisexualis*.

Constituent	% Dry Weight
1. Glucan ^a	52.80
2. Glucan ^b	50.23
3. Cellulose II	20.83
4. Alkali-soluble hexosamine	0.04
5. Insoluble hexosamine	2.59
6. Protein	10.00
7. Total amino acids	3.42
8. Uronic acids	0.03
9. Phosphorus	0.15
10. Readily extractable lipids	T
	86.44% ^c

^a determined (as glucose) with anthrone on unhydrolyzed walls; calculated as anhydroglucose

^b sum of acid- and alkali-soluble portions and the insoluble residuum of the wall

^c sum of 1, 3, 4, 5, 6, 8, and 9

Figure 9. Increase in reducing sugars of isolated wall after hydrolysis by *A. ambisexualis* cellulase (uncorrected data). (o, ... substrate; ●, ———, enzyme; Δ, — — — — —, enzyme-substrate.)

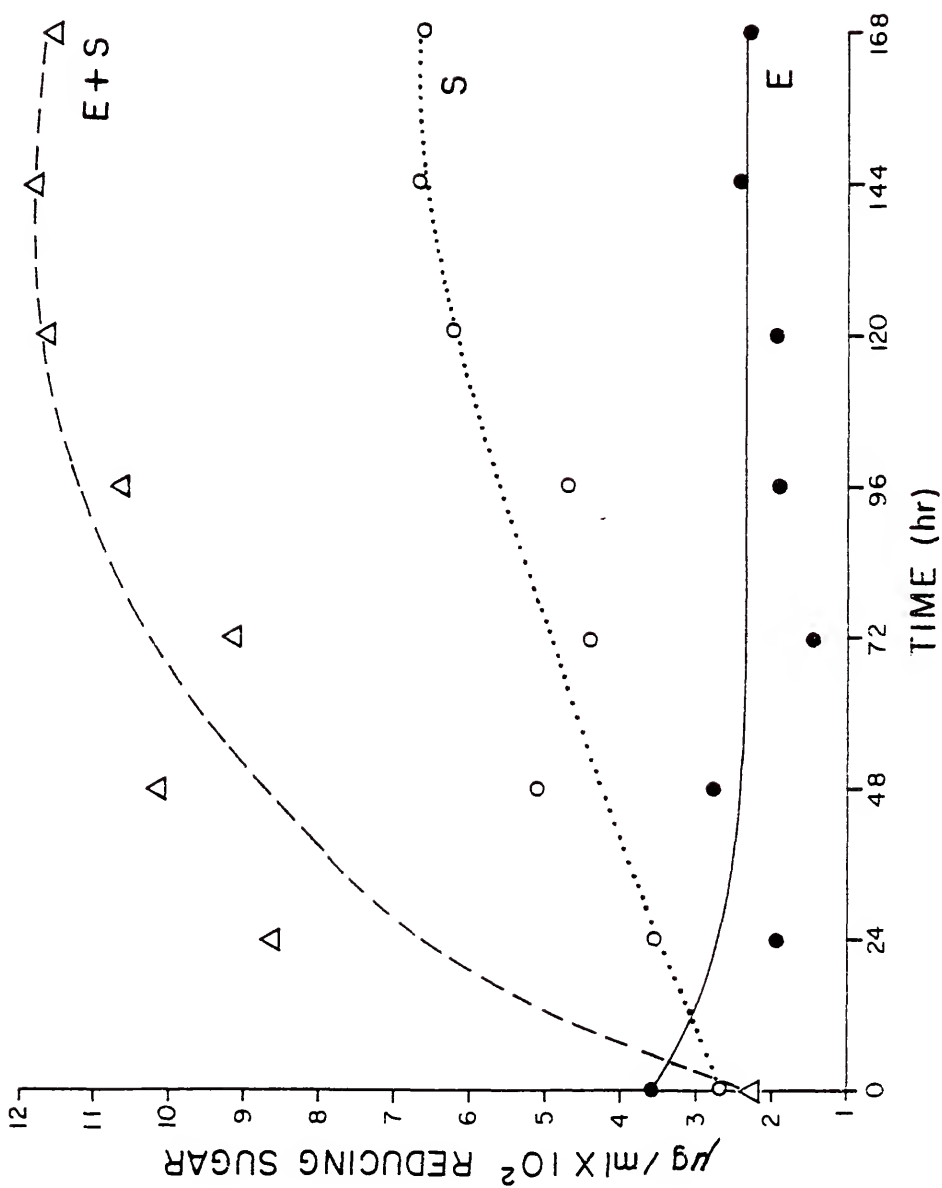


Figure 10. Increase in reducing sugars of isolated wall after hydrolysis by *A. ambisequalis* cellulase. Corrected for enzyme and substrate control backgrounds.

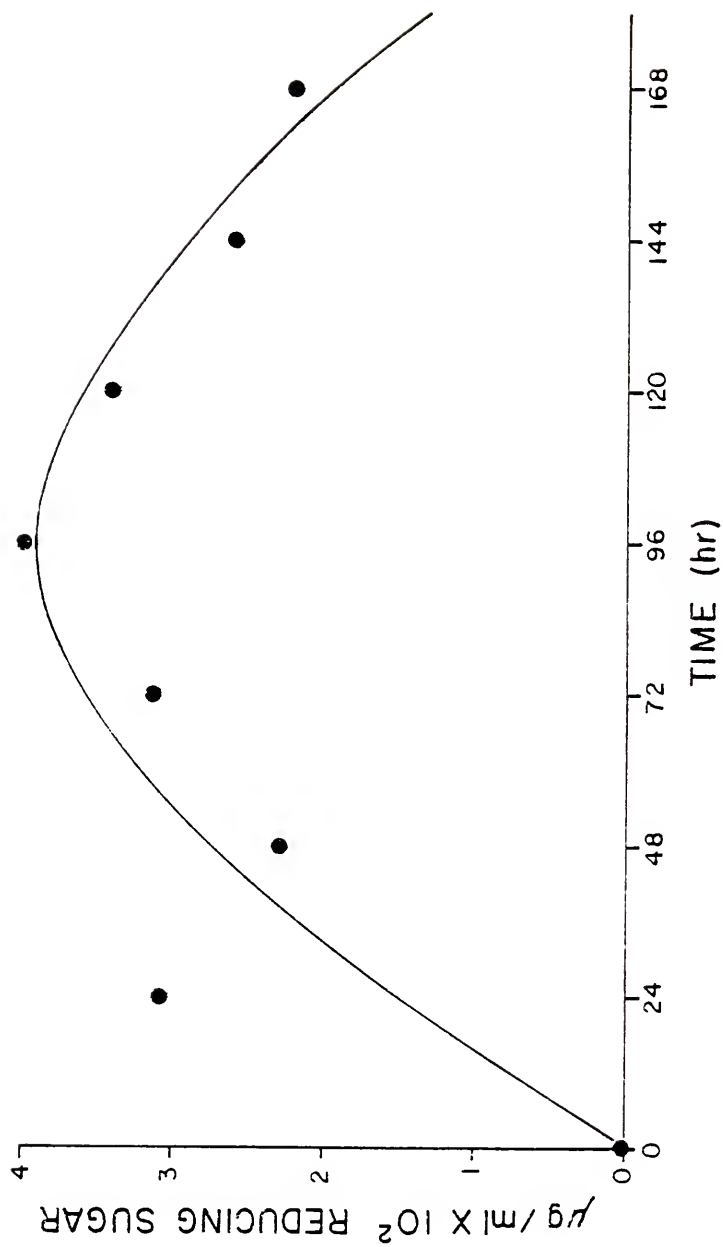
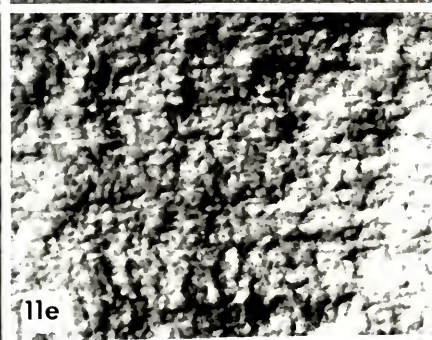
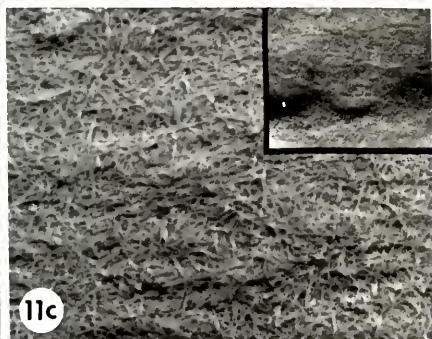
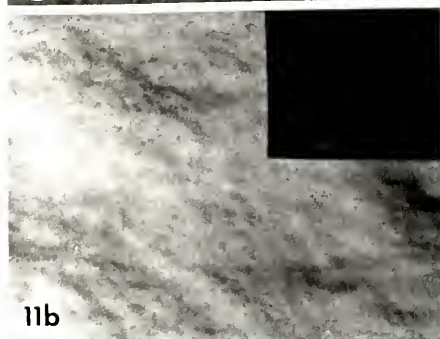
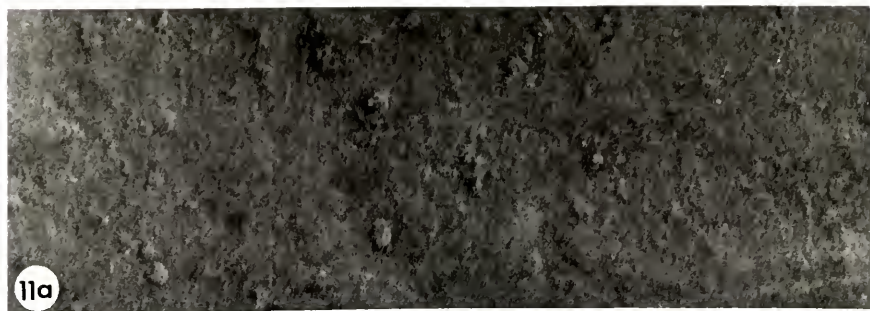


Figure 11. Surface replicas of isolated walls treated chemically. (a) Untreated. The surface is relatively smooth with a few microfibrils. X 26 000. (b) 0.5 N HCl treatment. Microfibrils are indistinct in both the control (inset) and the treated. X 30 000. (c) Treatment with 0.5 N HCl followed by 2 N KOH. Microfibrils, not seen in the control (inset), are evident after treatment. X 30 000. (d) Sequential treatment of 0.5 N HCl, 2 N KOH, and cadoxen. Control material (inset) appears intact and smooth, while in the treated only scattered pieces of amorphous material remain. X 27 000 (control), x 30 000 (treated). (e) Cadoxen-soluble wall material reconstituted. No recognizable microfibrillar pattern is seen. x 30 000.



untreated, but no microfibrillar structure is revealed (Fig. 11b). Walls treated with 0.5 N HCl followed by 2 N KOH show a pattern of microfibrils (Fig. 11c). Sequential treatment of walls with acid, alkali, and cadoxen results in a general disintegration with only pieces of amorphous material remaining (Fig. 11d). When the cadoxen-soluble material was reconstituted as cellulose II, a definite linear pattern resulted (Fig. 11e). This pattern does not resemble the microfibrillar one seen after acid and alkali treatment.

Surface Structure of Wall Fractions

A surface replica of the acid-soluble fraction has an amorphous appearance (Fig. 12a), while that of the alkali-soluble fraction appears weakly microfibrillar (Fig. 12b). The surface of the cellulose II fraction does not appear microfibrillar in Fig. 12c, but does in Fig. 12d. Cellulose I has a microfibrillar pattern similar to that seen after acid and alkali treatment (Fig. 12e). The insoluble residuum reveals faint microfibrils (Fig. 12f). The chitinous portion of the wall is microfibrillar, and it appears that the individual microfibrils are bound together in bundles (Fig. 12g). These bundles are arranged longitudinally, and there are a few microfibrillar groups which seem to run perpendicularly to the longitudinal ones.

Surface Structure of Chemically Treated Live Hyphae

The smooth surface of an untreated hyphae is seen in Fig. 13a. Mild acid (0.5 N HCl) treatment suggests an underlying pattern (Fig. 13b). Treatment with 0.5 N HCl followed by 2 N KOH produces a dramatic change with microfibrils becoming very evident (Fig. 13c). The surfaces of

Figure 12. Surface replicas of wall fractions. (a) Acid-soluble fraction. The surface is amorphous. X 30 000. (b) Alkali-soluble fraction. Some microfibrils are visible. X 30 000. (c) Cellulose II. No microfibrillar pattern is evident. X 32 000. (d) Cellulose II. Microfibrils are seen in apparent aggregations. X 32 000. (e) Cellulose I. Microfibrils are evident. X 32 000 (f) Insoluble residuum. A faint microfibrillar pattern is seen. X 32 000. (g) Chitin. Distinct arrangements of microfibrils are seen. X 32 000.

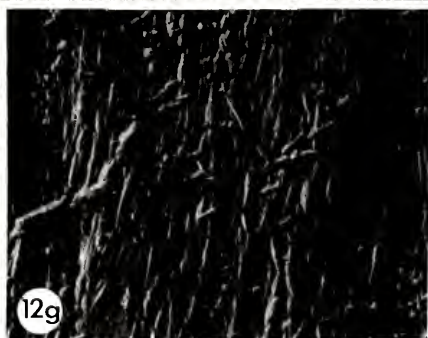
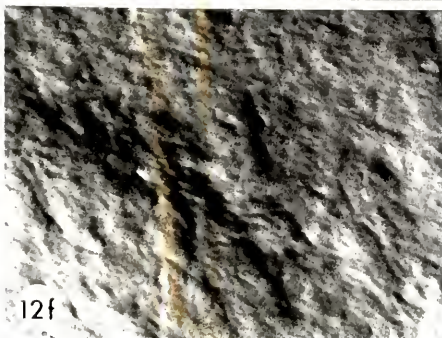
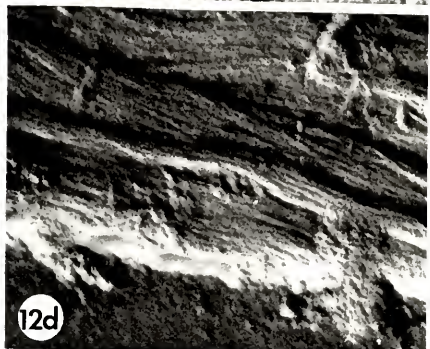
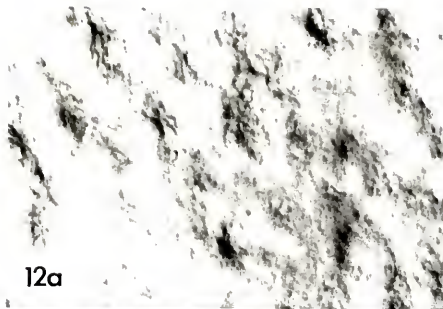
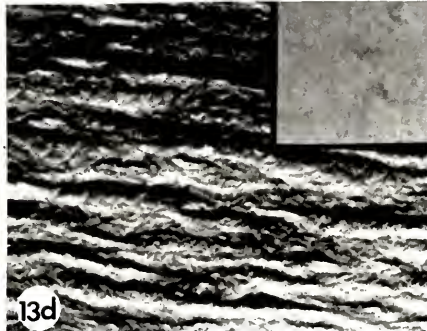
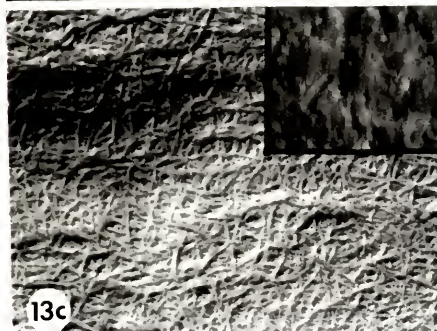
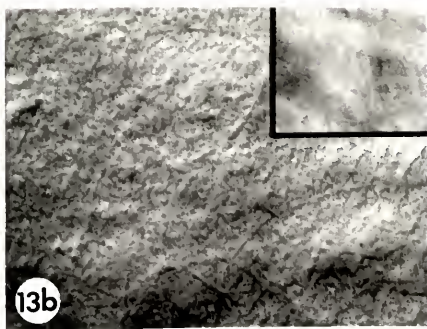
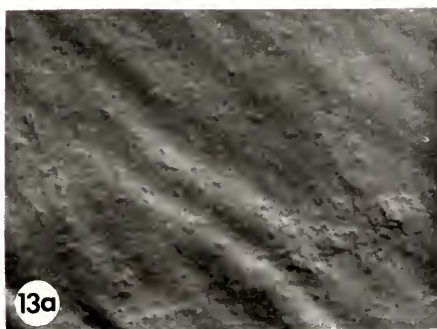


Figure 13. Surface replicas of live hyphae after chemical treatment. (a) Untreated. The surface is smooth. X 27 000. (b) 0.5 N HCl treatment. The surfaces of both the control (inset) and the treated samples appear amorphous. Faint microfibrils are seen in both samples. X 32 000. (c) Treatment with 0.5 N HCl followed by 2 N KOH. The control (inset) surface is amorphous, while that of the sample shows distinct microfibrils. X 30 000. (d) Sequential treatment of 0.5 N HCl, 2 N KOH, and cadoxen. The surface of the control (inset) is smooth, while that of the sample is striated with a suggestion of fibrillar material. X 30 000.



hyphae after acid, alkali and cadoxen treatment appear textured with a striated pattern and faint microfibrils can be seen (Fig. 13d).

Surface Structure of Enzymatically Treated Live Hyphae

Walls treated with laminarinase show distinct microfibrils while those treated with boiled enzyme resemble untreated walls (Fig. 14a). The cellulase prepared from *Aspergillus niger* or *Achlya ambisexualis* gives a smooth surface in both cases (Fig. 14b and c). The protease treated walls resemble their controls (Fig. 14d).

Sequential treatment with laminarinase and protease reveals microfibrils somewhat more sharply than treatment with laminarinase alone (Fig. 15a). Treatment with laminarinase followed by *A. niger* cellulase produces naked microfibrils (Fig. 15b). Laminarinase followed by *A. ambisexualis* cellulase results in a fragmented appearance with no long sections resembling hyphae remaining. The resulting pieces show scattered microfibrils (Fig. 15c).

Results from the sequential treatment with laminarinase, protease, and then cellulase from either *A. niger* or *A. ambisexualis* are given in Fig. 16a and b. The treatment containing cellulase from *A. niger* showed that drastic digestion had occurred and it was difficult to find structures resembling hyphae. When found, such pieces give the appearance of scattered microfibrils (Fig. 16a). Walls treated with the mixture containing *A. ambisexualis* cellulase reveal a pattern similar to that seen in the laminarinase plus *A. ambisexualis* cellulase treatment (Figs. 16b and 15c). Nothing resembling a hyphal structure was found.

Microfibrillar Diameter

The diameter of the few microfibrils detected in buffer treated samples was 11.23 nm while those of the laminarinase or

Figure 14. Surface replicas of live hyphae after single enzyme treatment. (a) Laminarinase. Microfibrils, not seen in the control (inset), are very evident in the treated sample. X 26 000. (b) *A. niger* cellulase. Both the control (inset) and the treated surfaces are amorphous. X 30 000. (c) *A. ambisexualis* cellulase. Both the control (inset) and the treated surfaces are amorphous. X 26 000 (control), X 30 000 (treated). (d) Protease. Both the control (inset) and the treated surfaces are amorphous. X 30 000.

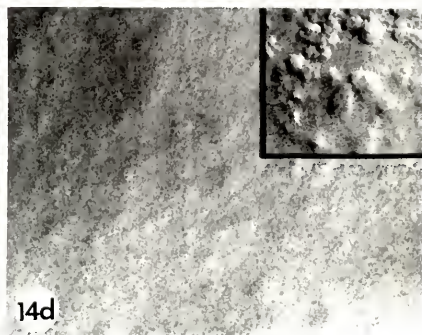
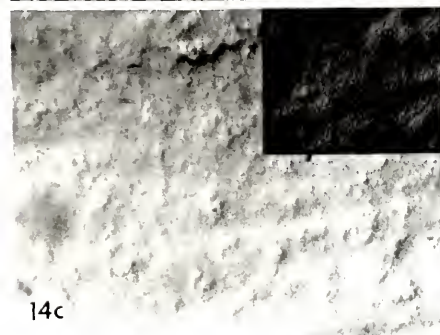
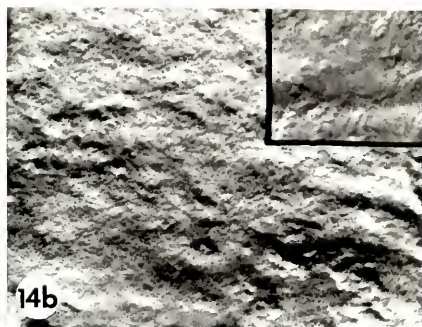
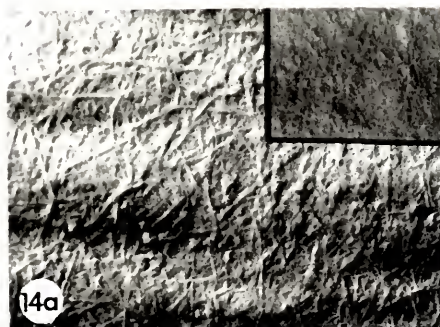


Figure 15. Surface replicas of live hyphae after sequential enzyme treatment. (a) Laminarinase and protease. The control (inset) appears amorphous while the treated is distinctly microfibrillar. X 30 000. (b) Laminarinase and *A. niger* cellulase. The control (inset) is smooth, but microfibrils are evident in the treated hyphae. X 26 400. (c) Laminarinase and *A. ambisexualis* cellulase. The control (inset) appears normal, but the treated hyphae are almost totally destroyed. One of the few remaining pieces shows some microfibrils. X 26 000 (control), X 30 000 (treated).

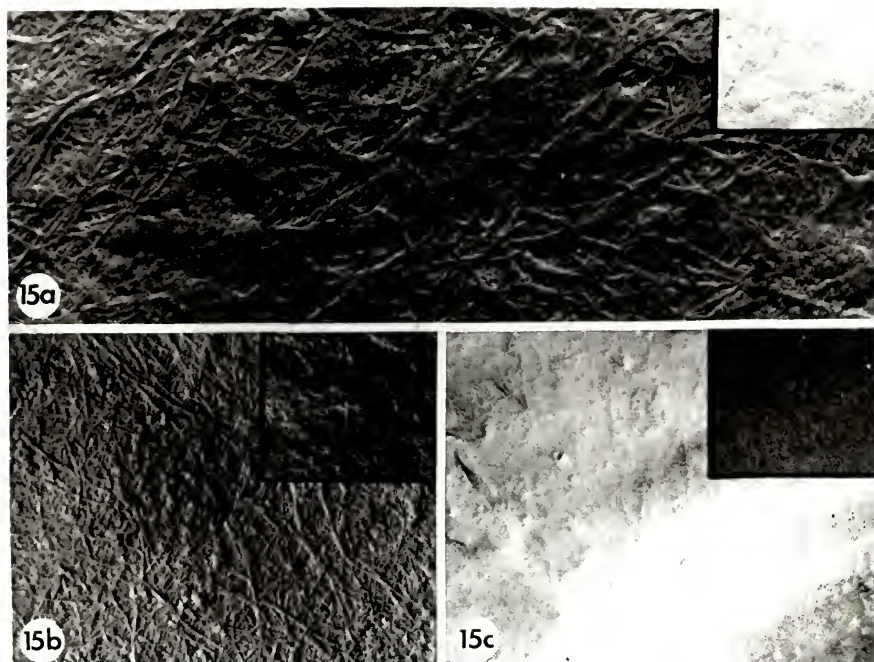
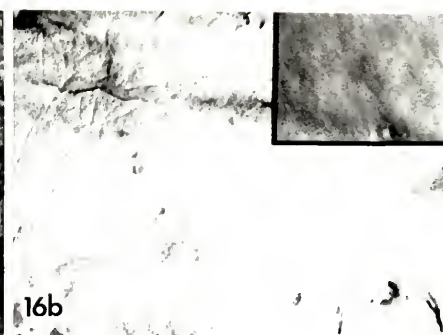


Figure 16. Surface replicas of live hyphae after sequential enzyme treatment. (a) Laminarinase, protease, and *A. niger* cellulase. The control (inset) appears amorphous. The treated hyphae are disrupted with only scattered patches of microfibrils remaining. X 30 000. (b) Laminarinase, protease, and *A. ambisexualis* cellulase. The control (inset) is smooth. The remaining pieces of the treated hyphae reveal few microfibrils. X 30 000 (control). X 24 900 (treated).



laminarinase-protease treated were 15.96 nm and 15.43 nm respectively (Table 12). The diameter of microfibrils revealed in hyphae treated with 0.5 N HCl and 2 N KOH was 20.29 nm (Table 12). Measurements of cellulose I microfibrils indicated that the width was 21.76 nm (Table 12).

Table 12. Microfibrillar diameter of various preparations from *A. ambisexualis*.

Treatment of Live Hyphae	Microfibrillar diameter ^a
Buffer	11.23
Laminarinase	15.96
Laminarinase-protease	15.42
0.5 N HCl- 2 N KOH	20.29
Wall fraction-Cellulose I	21.76

^anm; final number is average of between 20 and 60 measurements

DISCUSSION

The Preparation of Wall Samples

A comparison of the two methods of wall preparation reveals certain major differences in the chemical composition. A large discrepancy in total amino acids and amino sugars is evident (Table 9). The values for buffer-water washed walls were 3.42 mg amino acids and 1.22 mg amino sugars/100 mg dried walls, and for chemically cleaned walls 0.16 mg and 0.51 mg, respectively. The quality of amino acids was also different as judged by the lack of hydroxyproline, cysteine, and tyrosine in the chemically cleaned walls (Table 9). Proline was present in these preparations in only trace amounts. The combination of detergent treatment and extraction with ethanolic KOH in boiling water removed certain amino acids and perhaps proteins as well. This could be significant since protein may be a structural component of the wall (Hunsley and Burnett 1970; Wrathall and Tatum 1973). These results and others (Cameron and Taylor 1976) indicate that a given method of wall preparation clearly influences the values obtained.

Chemical Fractionation of the Wall

The fractionation procedure used in this study has been employed in analyses of the walls of *Atkinsiella dubia* (Aronson and Fuller 1969), *Pythium debaryanum* (Yamada and Miyazaki 1976), and two species of *Phytophthora* (Bartnicki-Garcia 1966). The value of 37.58% for the

acid-soluble portion of the wall in *A. ambisexualis* (Table 2) compares with 16.3% for *A. dubia* (2.90% had already been solubilized in boiling water) (Aronson and Fuller 1969) and 63% for both *Phytophthora* sp. (Bartnicki-Garcia 1966) and *P. debaryanum* (Yamada and Miyazaki 1976). The value of 7.07% for the alkali-soluble portion compares with 20% for *A. dubia* (Aronson and Fuller 1969) and *P. debaryanum* (Yamada and Miyazaki 1976), and 3.1% for *Phytophthora* sp. (Bartnicki-Garcia 1966).

In this study the first fraction which was removed from the wall was the acid-soluble one and this was the major wall component. Analyses of enzyme and acid hydrolysates of this fraction by paper (Tables 3 and 4) and gas chromatography (Figs. 3, 4, 5, and 6) gave laminaribiose and gentiobiose, thereby demonstrating the presence of both β 1,3 and β 1,6 linkages (Aronson *et al.* 1967). No cellobiose was found. The data on periodate consumption and formate liberation (Table 5) plus the solubility in water or dilute acid are consistent with a highly branched glucan structure (Sietsma *et al.* 1969). To account for the ratio of laminaribiose:gentiobiose found in the GLC analysis, a structure with a β 1,3 backbone with single glucose units linked through the sixth carbon atom occurring every fifth glucose residue is suggested. Glucans with single unit branches have been postulated for the walls of *S. ferax* and *D. sterilis* (Sietsma *et al.* 1969) as well as *Pythium* sp. (Eveleigh *et al.* 1968) and *Pythium acanthicum* (Sietsma *et al.* 1975). In *S. ferax* and *D. sterilis* this fraction makes up 43% of the wall (Sietsma *et al.* 1969). It is possible that the β 1,6 linked side chains are longer than one unit (Zevenhuisen and Bartnicki-Garcia 1969) or that parallel homopolymeric chains of β 1,3 and β 1,6 linkages are present. A purified

β 1,6-glucanase and a known β 1,6 glucan would be useful in testing this possibility. Several variations of this type of glucan have been suggested. In *Phytophthora* sp. and *Pythium* sp. the side branches are β 1,3 linked and a few β 1,4 linkages are also found in the acid-soluble complex (Novaes-Ledieu and Jiménez-Martínez 1969). In *Pythium debaryanum* the acid-soluble fraction is described as a β 1,3 branched glucan (Yamada and Miyazaki 1976). In *Phytophthora cinnamomi* the portion of the wall insoluble in Schweitzer's reagent (*i.e.*, the β 1,3 and β 1,6 glucans) is said to consist of a core of β 1,3 or β 1,6 or mixed linked main chains with short (four or five units long) β 1,3 linked side branches (Zevenhuisen and Bartnicki-Garcia 1969). A few β 1,4 linked chains are also thought to exist in this fraction (Zevenhuisen and Bartnicki-Garcia 1969).

The second fraction isolated from the wall was soluble in 2 N KOH. This fraction released glucose, laminaribiose, gentiobiose, and cellobiose upon acid hydrolysis (Table 4). Treatment with laminarinase released glucose and laminaribiose (Table 3). These results indicate three different linkages, β 1,3, β 1,4, and β 1,6. The high amount of periodate consumption relative to formate liberation plus the insolubility in water and dilute acid suggest that this component is rich in β 1,4 linked glucans (Table 5). The observation of faint spots representing gentiobiose also suggest β 1,6 linked units. A similar preparation from *P. acanthicum* also contained all three of these linkages (Sietsma *et al.* 1975), while the one from *P. debaryanum* had only β 1,3 and β 1,6 forming highly branched glucans (Yamada and Miyazaki 1976). The proportion of β 1,4 glucans in this fraction suggests that it may represent material from the cortex of the microfibrils, thought to consist of cellulosic

and hemicellulosic components thus differing from the cellulosic core (Preston 1974a).

The third fraction isolated was soluble in Schweitzer's or cadoxen reagent and is thus cellulose II, a β 1,4 glucan. Both x-ray diffraction analysis (Table 7 and Fig. 7), which is considered definitive evidence for cellulose (Weijman 1979), and polarizing light microscopy are indicative of cellulose II. The presence of glucose and cellobiose in acid hydrolysates of cellulose I, *i.e.*, not solubilized and regenerated (Table 4), provide additional evidence for cellulose. X-ray diffraction studies would be useful in confirming the identity of this component. The amounts of isolated cellulose I and solubilized cellulose II are similar (Table 2). When these two substances are compared to a cellulose standard by periodate oxidation they are similar (Table 5). Cellulose I liberated about twice as much formate as cellulose II or the known cellulose standard, indicating a shorter chain length. The presence of laminaribiose and gentiobiose (Fig. 4) indicated that the cellulose II fraction was not pure. This result is not surprising in light of Preston's (1974a) concept of microfibrillar structure.

The insoluble residuum is similar to the alkali-soluble component. A difference is seen in the ratio of periodate consumption to formate liberation (Table 5) suggesting that the polymeric chains of this fraction are longer than those of the alkali-soluble. Based on the fact that this fraction remains after acid, alkali, and cadoxen treatments, it is postulated that this residuum lies adjacent and is tightly bound to the plasmalemma. Its extreme insolubility may be due to extensive cross-linking of the polymeric chains and its function may

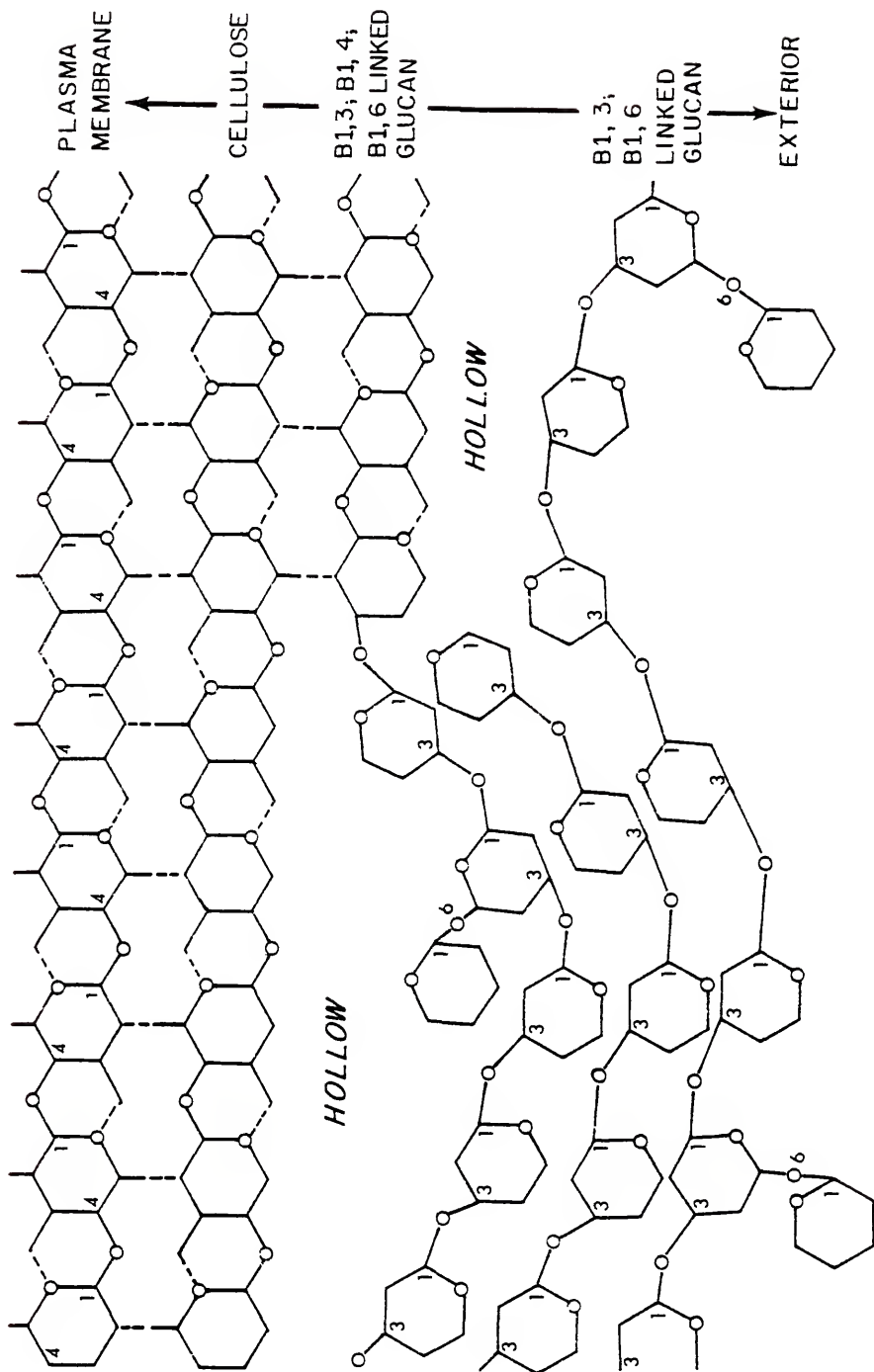
be protective. Or, it is possible that this fraction is part of the others and is the result of incomplete solubilization.

Analyses of enzyme and acid hydrolysates of the unfractionated wall (Tables 3 and 4, and Figs. 3, 4, 5, and 6) indicated the presence of a majority of β 1,3 and β 1,6 linkages and a minority of β 1,4. High periodate consumption and a moderate amount of formate liberation were seen (Table 5). It is hard to interpret this datum, especially when the ratio of laminaribiose:gentiobiose, as determined from the GLC data, is close to one, a deviation from that of the acid-soluble fraction. A possible explanation for the large periodate consumption as compared with formate liberation is the existence of β 1,4 linkages.

The linking and branching data have been used to prepare a model of the carbohydrate portion of the wall (Fig. 17). Speculation on the location of the protein component is also included in this model. A cellulosic backbone, hydrogen bonded both within and between polymers, is bound, again by hydrogen bonds, and possibly hydroxyproline-rich protein (Novaes-Ledieu and Jiménez-Martínez 1969), to the β 1,4 glucan portion of the alkali-soluble fraction of the wall. The β 1,3 glucan segment of the alkali-soluble fraction "nests" with like components of adjacent polymers forming a strong pliant structure. The outer β 1,3 linked glucans of the nest are part of the acid-soluble fraction of the wall and are characterized by the presence of β 1,6 linked branch points occurring about every fifth glucose residue. The function of uronic acids, the glucosamine units from the small alkali-soluble component (Table 8) (Novaes-Ledieu and Jiménez-Martínez 1969), and the β 1,6 linked branch points may be to serve as bridges between the polymeric components.

Figure 17.

Molecular model of the carbohydrate portion of the hyphal wall. ----- hydrogen bonds, both intra- and intermolecular. The areas labeled "hollow" are created by the nesting of adjacent linear β 1,3 glucans and are considered to be potential sites for hydroxyproline-rich proteins. The insoluble residuum is not included in this model, as its position in the wall is uncertain.



Chemical Analyses of Wall Constituents

Neutral Sugars

Comparison on a percentage basis of the chemical components of the *A. ambisexualis* wall with other members of the Saprolegniales indicates a fundamental similarity. Glucose was the only neutral sugar found in the *A. ambisexualis* wall. In general this is the pattern for other members of this order, although trace amounts of several other monomers have been reported (Crook and Johnston 1962; Parker *et al.* 1963; Novaes-Ledieu *et al.* 1967). While the total glucan of unhydrolyzed walls is 52.8% and of hydrolyzed walls, including the insoluble residuum, 50.23%, this does not account for the entire glucan component of the wall (Table 11). The cellulosic component of the wall is excluded from the above figures, as it has been found that the quantitation of glucan of this fraction cannot be determined by the anthrone method. This results from its extreme insolubility, even in the sulfuric acid medium of the anthrone reagent. If the assayable glucan and the cellulose portions are added together the wall consists of 73.63% glucan. This figure is in agreement with that found for *Saprolegnia diclina* (72.6%) (Cameron and Taylor 1976) and *Atkinsiella dubia* (78.4%) (Aronson and Fuller 1969), but is lower than that for some other species of *Saprolegnia* (Novaes-Ledieu *et al.* 1967; Sietsma *et al.* 1969). The cellulose content of *A. ambisexualis* walls is 20.83% which agrees quite closely with the 18% found in *Saprolegnia ferax* and *Dictyuchus sterilis* (Sietsma *et al.* 1969) and the 15% found in various species of *Saprolegnia*, *Achlya*, *Brevilegnia*, and *Dictyuchus* (Parker *et al.* 1963). Other workers

have found much higher amounts (42%) in the walls of *S. ferax* (Novaes-Ledieu *et al.* 1967).

Amino Sugars

The hexosamine content of the wall in *A. ambisexualis* is 2.6% (Table 8) and is similar to that found by Dietrich (1973) for three other species of *Achyla* and that found in *S. ferax* and *D. sterilis* (Sietsma *et al.* 1969). Studies of other members of this order revealed lower concentrations of amino sugars (Novaes-Ledieu *et al.* 1967; Cameron and Taylor 1976). Hexosamines are very susceptible to acid destruction as can be seen in the different amounts found in 4 N HCl (2.60% after 16 hours) and 6 N HCl (1.65% after 12 hours). Thus the actual amount of this sugar is probably higher than reported values. The hexosamine component in *A. ambisexualis* walls is glucosamine as identified by standards run with the samples in the amino sugar analyses.

Solubility in 1 N acids is one of the methods used to distinguish chitosan from chitin, the former being soluble while the latter is not (Muzzarelli 1977; Mendoza *et al.* 1979). Chitin is insoluble in both acid and base (Tracey 1955). The isolated chitinous component from *A. ambisexualis* walls is not chitosan based on solubility characteristics plus hydrolysis by chitinase. Lin *et al.* (1976) reported that in *Apodachyla* insoluble glucosamine is indicative of the presence of chitin in the wall. This was based on former studies identifying the presence of chitin by x-ray diffraction (Lin and Aronson 1970). Perhaps deacetylation occurred under the conditions of hydrolysis (Muzzarelli 1977) used prior to analysis of the insoluble glucosamine component and the glucosamine content recorded is really N-acetylglucosamine. Most

likely the chitin isolated from the wall (0.63 mg/100 mg dried walls) is part of the larger insoluble glucosamine component (2.59 mg/100 mg dried walls). The procedure for isolating chitin from the wall is quite extensive, and it is possible that a considerable amount was lost. Since the component from *A. ambisexualis* showed a positive reaction with Lugol's iodine, a test for chitosan, it appears to be a weakly acetylated chitin. X-ray diffraction analysis is necessary for the correct identification of this wall constituent. N-acetylglucosamine has been reported in the walls of *S. ferax* (Sietsma *et al.* 1969).

Uronic Acids

Uronic acids are labile in acids and are thus overlooked in some studies or reported at levels lower than the actual value (Rosenberger 1976). For this reason it is possible that the value (0.03%) found for *A. ambisexualis* walls is lower than the true value. Walls of *S. diclina* have been reported to contain uronic acids at the level of 1.3% (Cameron and Taylor 1976).

Protein and Amino Acids

The 10% protein found in *A. ambisexualis* walls is similar to the 8.5% found in *S. diclina* walls by Cameron and Taylor (1976). These figures are much higher than those found in earlier studies, *i.e.*, 3% in *Saprolegnia ferax* (Sietsma *et al.* 1969), except in the case of *A. dubia* where total protein was 13.7% (Aronson and Fuller 1969). The hydroxyproline content of the *A. ambisexualis* wall is only 0.1% (Table 9), although this is higher than the 0.02% found in *S. diclina* walls (Cameron and Taylor 1976). The value found for *A. ambisexualis* walls

may be lower than the true value because ninhydrin was used as the detector and this compound is known to underestimate the amounts of both hydroxyproline and proline (Tristram and Smith 1963). Separate analysis for this imino acid should be done in order to get a more reliable value. The presence of cysteine in *A. ambisexualis* at 0.04% (Table 9) is similar to that reported for *A. dubia* (Aronson and Fuller 1969). It was not found in *S. dielina* (Cameron and Taylor 1976). A comparison of the total content of amino acids (Table 9) and the total protein indicated a loss of 70% of the amino acids during hydrolysis and/or analysis. It was noted during the hydrolysis that humin and NH_3 were produced indicating destruction (Lippman *et al.* 1974; Taylor and Cameron 1973). It is also known that the predominance of polysaccharides in the reaction mixture will negatively influence the recovery of amino acids, except for serine and threonine whose stability is increased (Tristram and Smith 1963; Taylor and Cameron 1973).

Lipids

Only trace amounts of readily extractable lipids were found in *A. ambisexualis* walls. This differs from the 8.2% reported in *S. dielina* walls (Cameron and Taylor 1976). Other analyses have found between 1 and 5% in the walls of related organisms (Noveas-Ledieu *et al.* 1967; Sietsma *et al.* 1969). The trace of lipids found in this study was separated by thin layer chromatography and upon staining gave a positive test for phosphorus. Thus they may be phospholipids and the result of membrane contamination. Bound lipids were not extracted in this study but they were from the walls of *S. dielina* and found at the level of 3.75% (Cameron and Taylor 1976). Why there is such a wide discrepancy

in the amounts of this component in the walls of closely related organisms is not known, unless it is an artifact of preparation.

Total Wall Composition

The remaining problem in these analyses of the wall components of *A. ambisexualis* is the unidentified 13.5% (Table 11). Part of this unidentified fraction probably represents loss of material during hydrolysis. Another portion may be bound lipid and some may be ash. The ash component was not identified beyond phosphorus, which was analyzed and accounted for 0.15% of the wall. Ash content of closely related species include *S. ferox* at 3.2%, *D. sterilis* at 2.1% (Sietsma *et al.* 1969), *S. diclina* at 2.48% (Cameron and Taylor 1976), and *Atkinsiella dubia* at 1.5% (Aronson and Fuller 1969).

Hydrolysis of Buffer-Water Washed Walls by *A. ambisexualis* Cellulase

A study of the digestion of isolated walls by *A. ambisexualis* cellulase indicated that over a limited period of time there was an increase in reducing sugars produced (Figs. 9 and 10). This enzyme is active against carboxymethylcellulose and therefore is classed as an endo-enzyme (Reese and Mandels 1963a). It is evident from the description of the cellulase from *Aspergillus niger* that there are several β -glucosidases in the preparation (Hirayama *et al.* 1976), but the *A. ambisexualis* cellulase is reported to lack exocellulase and β -glucosidase activity (Thomas and Mullins 1969). The leveling off of enzyme activity seen here, even though visible pieces of wall remained in the reaction solution (Fig. 9), corroborates the supposition that the *A. ambisexualis*

cellulase is an endo-enzyme. The reduced level of substrate is thought to be responsible for the reduction of enzyme activity (Reese and Mandels 1963a). For the total enzymatic hydrolysis of cellulose the combination of endo- and exo-glucanases and β -glucosidases is required; the endo-enzyme is necessary for the generation of short pieces of cellulose on which the other enzymes can act (Okazaki and Moo-Young 1978; Ghose and Bisaria 1979). In the consideration of the activity of any cellulase knowledge of the structure of the cellulosic substrate is important. Such characteristics as degree of polymerization, substitution, porosity, and the distribution of crystalline and amorphous regions are important in determining the level of activity of a reacting cellulase (Cowling 1963; Berg 1978; Sasaki *et al.* 1979; Fan *et al.* 1980).

Ultrastructural Studies

Surface Structure of Enzymatically Treated Live Hyphae

Replicas of *A. ambisexualis* cellulase-treated live hyphae indicate no change in 48 hr of incubation (Fig. 14c), but treatment of live hyphae with laminarinase followed by *A. ambisexualis* enzyme show that drastic hydrolysis has taken place (Fig. 15c). No long hyphae were seen in a thorough examination of the replicas; only pieces remained. It seems as if once the matrix is removed the enzyme is able to hydrolyze the wall quite effectively, much better in fact than the *A. niger* cellulase (Fig. 15b). The fact that the *A. ambisexualis* enzyme is more effective than the one from *A. niger*, even though the latter appears more active based on viscometric data, may be because the former is adapted to hydrolyze the wall from within prior to lateral branch induction (Thomas and Mullins 1969).

The effect of protease is interesting. It does appear that there is a slightly better resolution of the microfibrils in live hyphae treated with laminarinase followed by protease (Figs. 15a and 14a), thereby confirming the observations made by Hunsley and Burnett (1970). Hyphae treated with the sequence of laminarinase-protease-*A. niger* cellulase appear to be almost totally destroyed (Fig. 16a). It would seem from these data that the cellulosic-protein linkages proposed by Hunsley and Burnett (1970) may in fact occur, as microfibrillar aggregations were not touched by the sequential application of laminarinase-*A. niger* cellulase but were, once protease was added. The "ghosts" or proteinaceous shells described by Hunsley and Burnett (1970) in *Phytophthora* after laminarinase-cellulase treatment were not observed here after similar enzyme application (laminarinase-*A. niger* cellulase) (Fig. 15b). Wall destruction is seen as the result of hydrolysis of the protecting protein by the protease followed by cellulase hydrolysis of the cellulosic component. Whether or not the proteinaceous shell of Hunsley and Burnett (1970) is the same as the protective proteinaceous component described here is unknown. In the case of laminarinase-*A. ambisexualis* cellulase treatment, total hydrolysis occurs without added protease. It must be remembered, however, that this enzyme may contain protease activity itself, as this type of contamination is commonly found in crude cellulase preparations (Whitaker 1970). The contaminating protease activity had been removed from the *A. niger* cellulase.

It would appear from these studies that the bonds of interest, those responsible for wall integrity, lie in both the matrix and the microfibrillar components, as laminarinase-*A. ambisexualis* cellulase or laminarinase-protease-*A. niger* cellulase are required for hyphal

demise. However, though single enzyme treatments do not seem to affect hyphal morphology, one cannot be sure that the cellulosic component was reached by the externally applied cellulase because of the protective matrix cover. Therefore, it cannot be ruled out that the bonds of interest may lie solely in the cellulosic component of the wall (Mullins 1979). In addition, it must be remembered that cellulase, produced by the organism itself, hydrolyzes from the inside. If the above morphological data are true, the enzyme acts directly on the cellulosic portion of the wall during the process of wall softening prior to branching. Therefore, it would seem that wall integrity, at least in branching, lies in the microfibrils or between the microfibrils and another component (Mullins 1979), perhaps protein. It is felt in general, though, that both the matrix and microfibrillar components are necessary for the maintenance of integrity (Bartnicki-Garcia and Lippman 1967; Hunsley and Burnett 1970); however, this conclusion is based on studies of wall hydrolysis of externally applied enzymes.

Surface Structure of Chemically Treated Walls and Live Hyphae

Treatment of isolated walls and live hyphae with mild acid demonstrates in both cases that the material (the acid-soluble fraction) which was removed does not itself directly cover the microfibrillar component of the wall (Figs. 11b and 13b). However, subsequent treatment with 2 N KOH removes the material (the alkali-soluble fraction) which does directly cover the microfibrils, as they are revealed with clarity comparable to the laminarinase-protease treatment (Figs. 11c, 13c, and 15a). Walls which were treated with acid, alkali, and cadoxen appear almost totally disintegrated (Fig. 11d), while live hyphae treated similarly retain

their morphology and have a striated appearance (Fig. 13d). The surface seen here may be that of the insoluble residuum described previously. The cadoxen-soluble material from the treatment of isolated walls was regenerated and it does not show the normal microfibrillar pattern seen after acid-alkali treatment (Fig. 11e). This material is comparable to the cellulose II fraction, in which some material has reformed into recognizable linear microfibrils (Fig. 12d).

Surface Structure of Wall Fractions

Surface replicas of the acid-soluble fraction appear amorphous, and it may be this fraction which is seen on the surface of untreated walls (Fig. 12a). The alkali-soluble fraction shows some microfibrillar structure which may be caused by the long stretches of β 1,4 linked glucose residues thought to exist in this component (Fig. 12b). This fraction may be comparable to the "short fibrils" seen after extensive exo- β 1,3-glucanase treatment by Sietsma *et al.* (1975). The insoluble residuum material appears weakly microfibrillar (Fig. 12f). Its insolubility in cadoxen may be because the β 1,4 linkages are interspersed with other linkage groups.

Microfibrillar Diameter

The measurements (Table 12) made in this study are similar to those of ethanolic-KOH cleaned walls (10-15 nm) (Tokunaga and Bartnicki-Garcia 1971) and untreated cyst walls (12-17 nm) (Desjardins *et al.* 1973) in *Phytophthora*. A discrepancy is seen in the measurements from the isolated microfibrillar portions of *Phytophthora* walls (Hunsley and Burnett 1968). The value reported is 13 nm for the microfibrils from distal walls (lower values were obtained for apical walls). It is thought that the apparent

increase in microfibrillar diameter seen in cellulose I could be due to the increasing dissolution of the matrix material thus revealing more and more of the microfibril (Fig. 18) or to acid swelling during the preparation.

There are criticisms of measuring microfibrillar diameter from shadowed material because of the variable amount of deposit added during the shadowing procedure (Preston 1974b). Microfibrillar diameters of material which have been negatively stained are much lower, as seen in the 3 nm widths of the microfibrils of *Aphanomyces astaci* (Nyhlén and Unestam 1978). A criticism of this method is that the stain penetrates the cortex area of the microfibril and therefore the only portion that is measured is the core (Preston 1974b). It has also been suggested that nascent microfibrils are wider than those after dehydration (Leppard *et al.* 1975).

Based on ultrastructural studies, particularly those using live hyphae where it is easier to determine which side of the wall is being acted upon, the amorphous layer hydrolyzed by laminarinase or dilute acid exists as the outer boundary of the wall. Moving inward the next layer is also hydrolyzed by laminarinase or alkali and appears to have an outer amorphous region and an inner one with a weakly microfibrillar pattern. The innermost layer of the wall is made up largely of cellulosic microfibrils which may be cross-linked with protein. The insoluble residuum remaining after wall fractionation is weakly microfibrillar and may be found between the plasma membrane and the microfibrillar portion of the wall. The idea of Phycomycete walls consisting of an amorphous outer layer and an inner microfibrillar one is not new

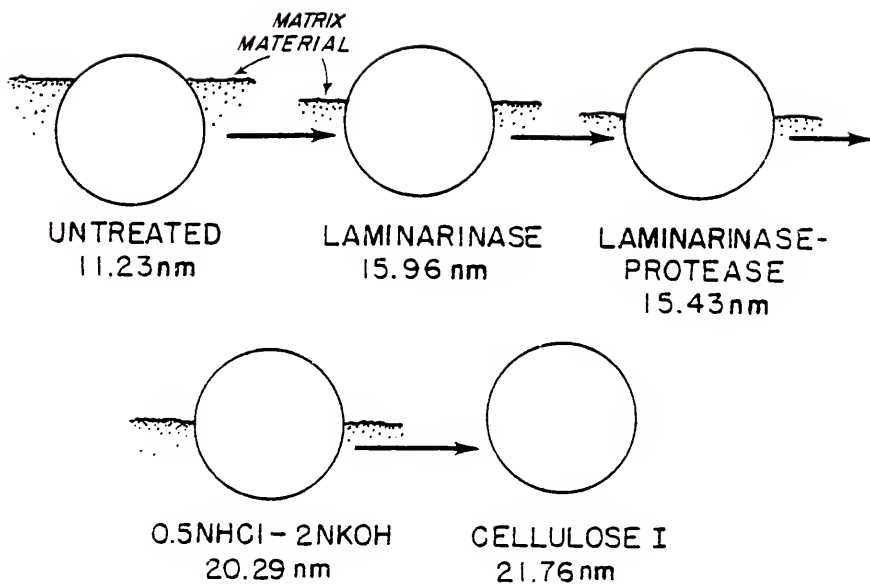


Figure 18. Scheme for explaining the apparent increase in microfibrillar width as a result of enzymatic or chemical treatment.

(Hunsley and Burnett 1970; Tokunaga and Bartnicki-Garcia 1971; Sietsma *et al.* 1975). Ultrastructural data suggest two layers for *Phytophthora* walls (Hunsley and Burnett 1970), but a more accurate description for *A. ambisexualis* walls might be two layers with a gradual change from one to the other as seen in the alkali-soluble fraction. A gradual change in wall layers in Phycomycetes has been suggested by Bartnicki-Garcia (1973). A schematic drawing of the layers of the hyphal wall, demonstrating the gradual change from one to the next, is seen in Fig. 19.

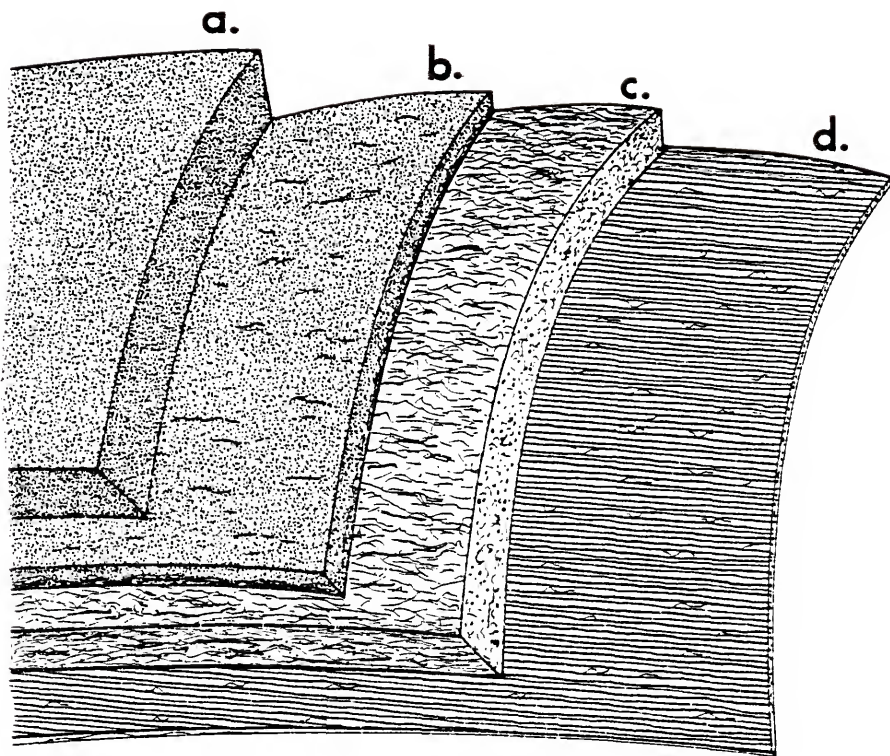


Figure 19. Diagrammatical representation of the hyphal wall based on ultrastructural evidence. (a) Acid-soluble and laminarinase hydrolyzed. (b) Alkali-soluble and laminarinase hydrolyzed. It should be noted that the upper side, corresponding to the outer portion, is fairly similar to (a), but that there is a gradual change to a more fibrous condition, similar to (c), on the lower side. (c) Cadoxen-soluble, and cellulase and protease hydrolyzed. Distinct microfibrils can be seen. (d) Insoluble residuum. The exact location of this fraction is uncertain, but it is thought that it may lie next to the plasma membrane.

CONCLUSION

In conclusion this analysis of the hyphal wall of *A. ambisexualis* has confirmed the results of previous studies on other members of the Phycomycetes. Some of the chemical differences noted may be the results of different procedures rather than actual variations between the walls themselves. Chemical fractionation of the carbohydrate wall constituents yielded four fractions based on solubility characteristics. The major component, soluble in weak acid, was found to consist of a β 1,3 glucan with numerous β 1,6 branch points. A smaller component, soluble in alkali, was determined to be a linear glucan with mixed β 1,3 and β 1,4 linkages with occasional β 1,6 branches. The microfibrillar component, soluble in Schweitzer's or cadoxen reagents, was examined and found to be cellulose. After these treatments, a small portion of mixed β 1,3 and β 1,4 linkages with a few β 1,6 remained. It is speculated that this material lies adjacent to the plasma membrane. Protein was also found and amino acid analyses revealed the usual spectrum of amino acids, including hydroxyproline, commonly found in cellulosic cell walls. The only known report of the probable existence of a chitinous wall component (Dietrich 1973) was confirmed.

Morphologically, the wall consists of an outer matrix of β 1,3 and β 1,6 glucans covering an inner cellulosic-proteinaceous core. A diagrammatic model (Fig. 19) synthesized from the chemical and morphological data is proposed. In addition, a molecular model (Fig. 17) of the hyphal wall, utilizing the data from the extensive study of the linkage and branching pattern of the wall fractions, is presented.

Based on the enzymological studies performed here, it would seem that the bonds which are necessary for wall integrity lie in both the matrix and the microfibrillar regions. However, the enzymes were applied from the outside, so it is possible that the microfibrillar component was never reached by the cellulase. Thus the bonds of interest may be in this constituent, or between it and another, possibly protein.

APPENDICES

APPENDIX A TECHNIQUES

Buffer-Water Washing of Isolated Walls (Lin *et al.* 1976)

Isolated walls were washed with 0.1 N tris-HCl buffer six times with low speed (1085 x *g*) centrifugations in between. The pellet, resuspended in 20 ml of the same buffer, was sonicated for 4 min at 30 watts. After sonication the pellet was washed six more times with the buffer, homogenized with a glass tissue grinder, and washed twelve times with distilled water or until the walls appeared clean microscopically and the supernatant from the washings was clear. Walls were then lyophilized and stored over desiccant.

Chitin Isolation (Aronson and Lin 1978)

Either lyophilized chemically cleaned walls (100 mg) or frozen mycelia (20 gm fresh weight) were used for this extraction. If the latter was used, it was homogenized with a mortar and pestle in 5% KOH in 80% methanol and placed at 98°C for 15 min. The resulting solution was centrifuged at low speed (1085 x *g*) and the pellet was treated as above two more times. The residue was washed twice with distilled water, once with 0.5 N acetic acid, and twice more with distilled water. It was then treated five times with 1 N acetic acid at 98°C for 15 min each time; the remaining residue was washed with water until it was acid free. Chitin was extracted from chemically cleaned walls or from mycelia prepared as above by the following procedure: treatment

with 50 ml 2% (w/v) KMnO_4 at 25°C for 18 hr with intermittent stirring followed by centrifugation and washing twice with distilled water and three times with 2% (w/v) oxalic acid containing a few drops of 1 N H_2SO_4 . Five additional washings were done. The residue was then stirred continuously under N_2 for three 1 hr treatments at room temperature in Schweitzer's reagent; the remaining residue ("chitin") was washed four times with 1 N acetic acid and then with water until acid free. The material was freeze dried and stored over desiccant until further analysis.

Cellulose I Isolation (Aronson and Lin 1978)

Cellulose I was extracted from chemically cleaned walls or from mycelia prepared as above (see Chitin Isolation) by treatment with 1 N KOH at 25°C for 1 hr, followed by three water washings and treatment with 1 N acetic acid at 98°C for 15 min followed by three more water washings. The alkali extraction and water washings were repeated and the residual material was suspended in H_2O_2 -acetic acid (equal volumes of 30% H_2O_2 and glacial acetic acid) and placed at 98°C for 30 min and then washed with distilled water three times. The insoluble material was treated with 500 $\mu\text{g/ml}$ chitinase in 0.05 M potassium phosphate buffer at pH 6.0 for 72 hr at 25°C with shaking. The remaining pellet was washed three times with water and stirred for 90 min in 5.25% NaOCl with 5% KOH. The pellet was then washed once with the chlorox solution, once with distilled water, once with 1 N acetic acid, and three times with distilled water. Then it was lyophilized and stored over desiccant.

Preparation of Acid Swollen Cellulose
(Green 1963; Reese and Mandels 1963b)

A 500 mg sample of Whatman No. 1 cellulose powder was treated with 15 ml 85% H_3PO_4 and stirred occasionally with a glass rod over a period of 65 hr. After this period of time all the cellulose appeared dissolved; five volumes of distilled water were added. This solution was filtered leaving a gelatinous mass on the filter paper which was washed twice with 95% ethanol and twice with xylene and dried overnight in a vacuum desiccator. This process is called WAN drying (water/alcohol/non-polar hydrocarbon).

Enzyme Purification (Sietsma *et al.* 1968)

Two enzymes, laminarinase and cellulase, were found to be impure and were subjected to the following purification process. Degassed, washed DEAE-cellulose (medium mesh, capacity 0.94 meq/gm, Sigma) was poured into a 23 cm column (I.D. 1.5 cm) and the enzyme solution to be purified (1 mg/ml in 0.005 M potassium phosphate buffer pH 7.5) was layered on top. The enzymes were eluted with a linear gradient of increasing NaCl concentration (0.0 to 0.8 M) in phosphate buffer. The salt concentration was measured by conductivity. Preliminary studies indicated that in the purification of cellulase most of the enzyme activity was found in the fractions which were eluted with a lower salt concentration; the enzyme eluted with 0.4 M salt was free of proteolytic activity. It was decided to use a stepwise elution process when it was found that the contaminating proteolytic enzymes were eluted at 0.2 M NaCl concentration. Thus two eluants were used, 0.2 M and 0.4 M NaCl in equal proportions. The 0.2 M fraction and the first 10% of the 0.4 M

were discarded. The remaining 0.4 M was saved and dialyzed for 48 hr against a 0.05 M sodium citrate buffer pH 5.0 (the buffer was changed six times during the dialysis period) to remove salt and to place the enzyme in an appropriate buffer for reaction. The enzyme was concentrated with an immersible molecular separator (Millipore Corporation) with a pellicon membrane of 10 000 nominal molecular weight limit or over Ficoll or carbowax. In general purified cellulase had an activity of about 12 units/ml.

The purification of laminarinase was carried out in the same manner. It was found that the bulk of laminarinase activity came out in the fractions eluted with salt concentrations lying between 0.25 M and 0.35 M. However, the fractions with salt concentrations in the vicinity of 0.35 M also contained cellulase activity, so it was decided, since more laminarinase activity was found in the 0.25 M fraction, to use only the enzyme from this fraction. From the above information it was decided that purified laminarinase could be obtained by eluting the column with 0.25 M NaCl. Dialysis and concentration of the 0.25 M fraction was the same as for the purified cellulase.

Enzymatic Hydrolysis of Laminarin

Two different sources of laminarin were used as substrates for the generation of β 1,3 linked di- and trisaccharides to be used as reference compounds for gas and paper chromatographic studies. Briefly, the procedure involved treating each of the substrates with purified laminarinase and incubating the reaction mixture at 37°C for 6 hr in 0.05 M citrate buffer pH 5.0. Undigested material was pelleted and aliquots of the supernatant were used as reference compounds. It was found that

the Calbiochem laminarin contained β 1,3 linkages while the other polysaccharide (source unknown) contained both β 1,3 and β 1,6.

Hydrolysis of the Unfractionated Wall with H_2SO_4

A 20 mg sample of dried walls was treated with 0.4 ml of 12 N H_2SO_4 under N_2 for 12 hr at room temperature. The acid solution was diluted to 1 N and placed at 105°C for 4 hr. Then it was diluted 10-fold and neutralized with BaOH , and the BaSO_4 precipitate was removed by centrifugation. The resulting supernatant was lyophilized and the residue was redissolved in 2.5 ml distilled water and after derivatization was analyzed by gas chromatography (personal communication Dr. R. Michael Roberts).

Description of Analyses Used for the Detection of Neutral Sugars

Paper Chromatography

The technique of paper chromatography involves the separation of substances based on their relative solubilities in water and an organic solvent (Kowkabany 1954; Bloch *et al.* 1958). The filter paper is a support for the water-rich stationary phase over which runs the organic solvent, the mobile phase. The substances to be analyzed were spotted 6.35 cm apart on a line near the top of the paper in concentrations of about 100 μg . The concentration of the standards was 50 μg . The spotted paper was hung from a trough in a presaturated chromatocab; 40 ml of the solvent was poured into the trough and the separation was run for 24 hr. At the end of this time, the chromatograms were removed from the chamber, dried in a hood, sprayed with an aniline phthalate spray for the detection

of reducing sugars, and developed in a 105°C oven. Unknown spots were identified by their similarity to R_f or R_g values of known standards. R_f is defined as the ratio of the movement of the spot to the movement of the solvent front; R_g is the ratio of the movement of the spot to that of known glucose and is more reliable if the solvent has run off the paper. For sugars the best resolution of separation occurs with solvents which give R_f values between 0.2 and 0.3. The butanol:pyridine: water solvent chosen gave the best results of those tried. Other solvents were 5 ethyl acetate:5 pyridine:1 acetic acid:3 water v/v, 2 ethyl acetate:1 pyridine:2 water and 8 ethyl acetate:2 pyridine:1 water.

Gas-Liquid Chromatography

The principle of gas-liquid chromatography is basically the same as that of paper. Substances are separated into their component parts between the mobile gas phase and the stationary liquid phase based on their partition coefficients (Bishop 1964; White *et al.* 1964; McNair and Bonelli 1968). The solubility of substances is dependent on many factors such as molecular weight, degree of substitution of side groups, polarity, and stearic factors. The stationary liquid phase is spread thinly over an inert solid, the support phase, generally a silanized diatomaceous substance, and then the gas phase moves through the stationary bed (McNair and Bonelli 1968; Pierce 1968). The components to be separated are carried by an inert gas, the carrier gas, and the sample is partitioned as described above. The separated samples leave the column in the carrier gas and in this case are sensed by a flame ionization detector (FID). The FID consists of a mixture of hydrogen and air which

produces a flame over which is placed an electrode which measures the conductivity of the flame. Pure hydrogen has low conductivity, but as the organic compounds in the carrier gas pass over the flame and are combusted there is an increase in conductivity which is amplified and recorded (McNair and Bonelli 1968). Partition coefficients are directly proportional to retention volumes, therefore the time at which a particular component comes out of the column is a good indication of its identity as compared with known standards. Temperature programming is a method by which an investigator can maximize separations and yet shorten the time substances remain on the column thereby obtaining peaks which are reasonably sharp. Generally, a temperature program must be arrived at empirically for there is much variation in the systems used and the components to be analyzed.

Derivatization of Samples

There are several ways of volatilizing carbohydrates for this type of analysis. The formation of trimethylsilyl groups was used in this study because of its ease and the relatively low number of components found in the substances to be separated. The reaction involved in the formation of these compounds is the replacement of the active hydrogen of the free hydroxyl group in polysaccharides by a silyl group; the use of both TMCS and HMDS insures that complete silylation will take place. Pyridine is used as a solvent (Pierce 1968). Hydroxyl hydrogens are replaced in order to prevent hydrogen bonding between free hydrogen groups and also to decrease the polarity of the compounds to be studied (Pierce 1968; Clamp *et al.* 1971).

Periodate Oxidation

Periodate oxidation studies were done in order to gain some knowledge as to the pattern of linkages and branching in the isolated fractions of the wall. The principle involved in these studies is that in long chain polymers there is under controlled conditions a specific pattern of periodate consumption and formate liberation which upon analysis gives information as to linkage and branching arrangements in the polymer (Hay *et al.* 1965). If the manner in which a chain is linked leaves two adjacent free hydroxyl groups per monomeric residue then it will take one molecular proportion of periodate to cleave the carbon chain and no formate will be released in the reaction. This is the case with glucan chains which are linked β 1,4. However, if the situation exists where there are three adjacent free hydroxyl groups, then it will take two molecular proportions of periodate to cleave the chain and one molecular proportion of formate will be released. This situation occurs at non-reducing terminal points and with β 1,6 linked non-terminal units. Periodate does not affect residues which joined such that there are no adjacent hydroxyl groups as is the case with β 1,3 linked glucans. It is not well understood what happens at the reducing end, but it is thought that two molecular proportions of formate are given off unless a formate ester is formed, in which case one proportion is given off (Bobbitt 1956; Smith and Montgomery 1956). Data from these studies can be seen in Tables 5 and 6, and Figs. 20, 21, and 22.

Polarized Light Microscopy

Polarized light microscopy is used to detect the presence of highly ordered systems, in this case cellulose. Polarized light, light vibrating

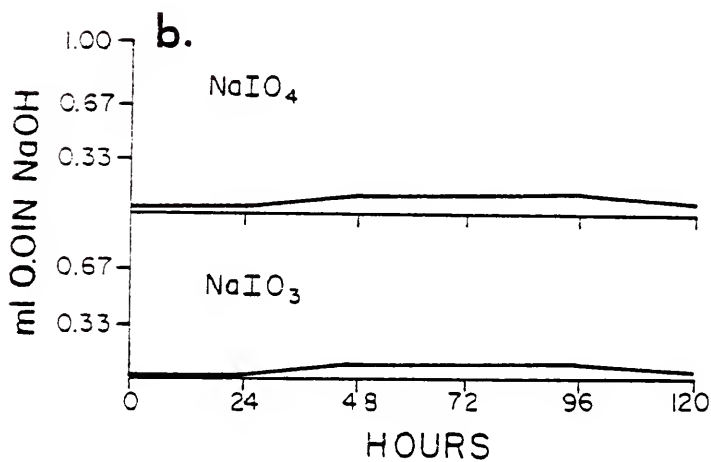
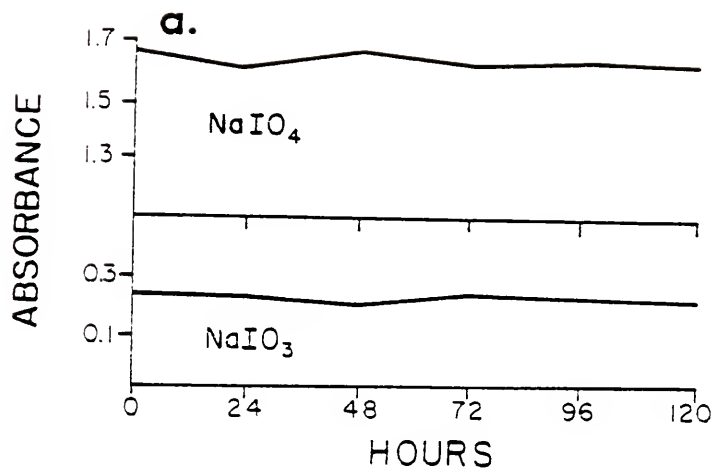


Figure 20. Periodate and iodate oxidation. (a) Periodate consumption. (b) Formate liberation.

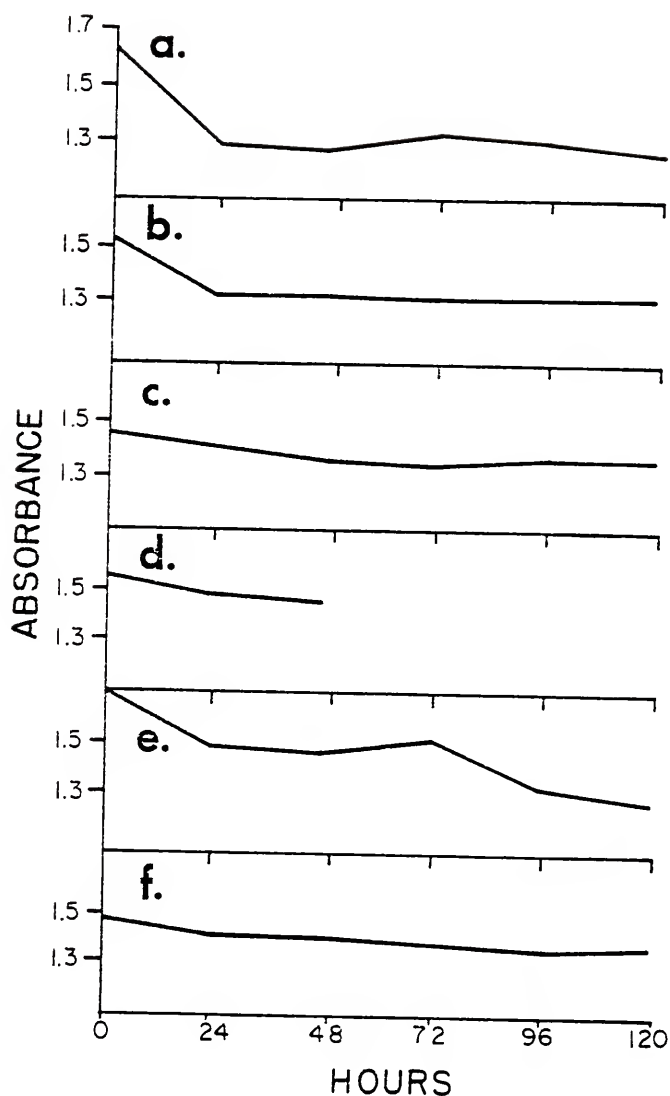


Figure 21. Periodate consumption of the wall fractions and the total wall. (a) Acid-soluble. (b) Alkali-soluble. (c) Cellulose II. (d) Insoluble residuum. (e) Total wall. (f) Cellulose I.

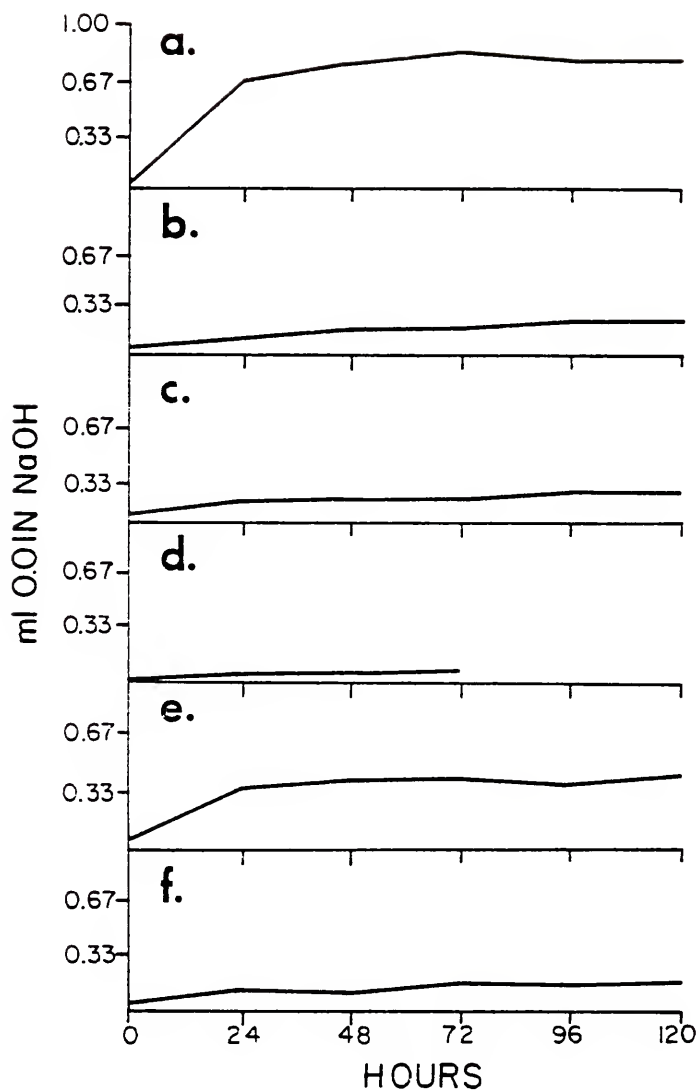


Figure 22. Formate liberation of the wall fractions and the total wall. (a) Acid-soluble. (b) Alkali-soluble. (c) Cellulose II. (d) Insoluble residuum. (e) Total wall. (f) Cellulose I.

in one plane, is "bent" to shine in a new plane by encountering solids of well-ordered molecular arrangements (Wolfe 1972). The phenomenon is known as birefringence.

X-ray Diffraction Analysis

X-ray diffraction analysis, by studying the angles and intensities of the scattering of x-rays at a given wavelength by the electrons which surround each atom, is used to measure the lattice spacings of crystal-line structures (Lehninger 1975). Atoms with higher electron densities produce more diffractions than those with lower densities and with this knowledge various patterns of atomic arrangement can be discerned. Photographs of x-ray diffraction studies can be made by placing the crystal in question in a known orientation in the path of monochromatic x-rays. The x-rays scattered by the crystal hit a photographic plate behind the crystal. The three dimensional structure of crystals can be obtained by doing a series of electron density photographs in different planes (White *et al.* 1964). Cellulose, having a well ordered molecular arrangement, is well suited to such studies.

Solubility Analysis of the Hexosamine Component of the Wall (Aronson and Lin 1978)

A 40 mg sample of buffer-water washed walls was treated with 4 ml 1 N NH_4OH for 8 hr at room temperature and then centrifuged; the pellet was treated again with 2 ml 1 N NH_4OH followed by a 2 ml water wash. All the supernatants were combined and dried *in vacuo*; these constituted the alkali-soluble portion of the hexosamine component. The remaining pellet was treated twice for 15 min with 4 ml 1 N acetic acid at 98°C, and once with 2 ml 1 N acetic acid at room temperature. Again all the

supernatants were combined and dried *in vacuo*; this was the acid-soluble hexosamine fraction. The pellet which remained was washed twice with 95% ethanol and dried under a stream of N_2 . All these fractions were hydrolyzed as described and analyzed for hexosamine content in an automated Amino Acid Analyzer.

Uronic Acid Analysis (Gancedo *et al.* 1966)

A 100 mg sample of buffer-water washed walls was hydrolyzed with 1% HCl for 2 hr in a boiling water bath. The hydrolysate was centrifuged and the supernatant was passed through an Amberlite (Mallinckrodt) IR-45 column to remove the mineral acid. About 65 ml was collected and this was run through a Dowex-I formate column which bound the uronic acid. The uronic acid was eluted with 0.5 N formic acid; 16 6 ml fractions were collected and analyzed for uronic acid content by the Carbazole test (Bitter and Muir 1962).

Lipid Extraction and Analysis (Whitehouse *et al.* 1958; Bartlett 1959; Ames 1968; Kanfer and Kennedy 1963)

A 40 mg sample of buffer-water washed walls was treated with 8 ml methanol and heated to 50°C for 30 min. The mixture was cooled to room temperature and 16 ml chloroform were added and the mixture was shaken for 30 sec. Then 24 ml 2N KCl were added, the mixture was shaken vigorously, and the phases were allowed to separate for 30 min. The top layer (methanol-salt) was aspirated off, distilled water added, and the solution was mixed. The phases were allowed to separate and again the top layer was aspirated off. The tubes were spun and the water layer (top) was removed; the remainder was dried under vacuum. The dried residue ("lipid") was spotted on Silica gel G plates as described previously.

Phosphorus Analysis (Bartlett 1959)

A 10 mg sample of buffer-water washed walls homogenized with a tissue grinder plus 0.5 ml 10 N H_2SO_4 was combusted in a 160°C oven for 3 hr, after which time 2 drops 30% H_2O_2 were added and the solution was returned to the oven. Periodically (every 3 hr or overnight) the solution was removed from the oven, treated with the H_2O_2 and returned to the oven. This was continued for 48 hr until the solution was clear and colorless, then a 5% urea solution was added and the tubes were combusted for 1 hr longer to insure that all the H_2O_2 was consumed (personal communication Dr. Thomas E. Humphreys). The Fiske-SubbaRow assay for phosphorus was performed on the resulting solution.

Ultrastructural Studies - Thin Section

Thin sections of untreated walls of live hyphae were made and measurements of the width of the wall were taken. Material was fixed in 5% glutaraldehyde for 1 hr and post-fixed with 1% osmium tetroxide for another hour. The specimens were dehydrated and embedded in Mollenhauer Mixture No. 2 (Mollenhauer 1964). Specimens were sectioned with a Huxley LKB microtome and placed on 100 mesh copper grids. They were then post-stained with 0.5% uranyl acetate and then treated with lead citrate. Microscopic observations were done with a Hitachi HU-11E electron microscope.

APPENDIX B
RECIPES

Growth Media for *A. ambisexualis*

Ingredient	Liquid gm/l	Agar (ENR)
		(Kane 1971) gm/l
Monosodium glutamate	0.4	0.5
Glucose	2.0	14.0
Tris (hydroxymethyl) aminoethane	1.2	1.2
Combined liquid stock ^a	17.5 ml/l	17.5 ml/l
Distilled water to	1.0 l	1.0 l
Agar	0.0	25.0
^a Combined liquid stock	ml/l	ml/l
l-Methionine (15 mg/ml in 10% HCl)	1.0	1.0
KCl (2 M)	1.0	1.0
MgSO ₄ ·7H ₂ O (0.5 M)	1.0	1.0
CaCl ₂ (0.5 M)	1.0	1.0
HEDTA (10 mg/ml)	2.0	2.0
KH ₂ PO ₄ (1 M)	1.5	1.5
Metal mix #4 (2 mg/ml) ^b	10.0	10.0
^b Metal mix #4; grind together		
Fe(NH ₄) ₂ ·(SO ₄)·6H ₂ O	28.9 gm	28.9 gm
Zn(SO ₄)·7H ₂ O	8.8 gm	8.8 gm
Mn(SO ₄)·H ₂ O	3.1 gm	3.1 gm

As used this metal mix provides Zn at 1.0 mg/l, Fe at 2.0 mg/l, and Mn at 0.5 mg/l.

Cadoxen Reagent (Jayme and Neuschaffer 1957; Jayme and Lang 1963)

100 ml 30% ethylenediamine
4 gm cadmium oxide (saturated solution)

The ethylenediamine-cadmium oxide solution was stirred under the hood at room temperature for 30 min. Excess cadmium oxide was removed by centrifugation (3020 x g, 10 min). The supernatant was used for cellulose dissolution; it was best when used freshly made.

Schweitzer's Reagent (Cuoxam Solution) (Jayme and Lang 1963)

54 gm C.P. copper (II)
10% NH_4OH
22% NH_4OH
Distilled water

The copper was heated to boiling in 300 ml distilled water. About 55 ml of the 10% NH_4OH was stirred in until a bluish color appeared. The precipitate was allowed to settle and the supernatant was decanted. The precipitate was washed with 3 or 4 100 ml portions of distilled water and the washings were decanted. The precipitate was rinsed with 22% NH_4OH into a dark bottle and diluted to one liter with 22% NH_4OH . The solution was stored at room temperature in the dark. The final concentration of copper was 13.5 gm/l.

Anthrone Reagent (Morris 1948; Dische 1962)

0.2 gm anthrone (Sigma)
100 ml concentrated H_2SO_4

To be prepared daily and chilled for 2 hr before use.

Material in the standard, blank, and sample tubes was brought to a final volume of 1.25 ml and chilled. Aliquots of 2.5 ml of the anthrone reagent were layered on and the tubes were covered with parafilm. The tubes were mixed with a vortex and chilled. The parafilm was then removed and a marble was placed on top of the tubes which were then put in a boiling water bath for 16 min. After removal from the boiling water bath tubes were set in an ice bath for 2 to 3 min and then allowed to stand at room temperature for 5 to 10 min. They were then read at 620 nm against a reagent blank in a Gilford 240 spectrophotometer; glucose was used as the standard.

Glucostat Test (Worthington Biochemical Corporation, Freehold, NJ 07728)

Principle: β -D-glucose + O₂ + H₂O $\xrightarrow{\text{glucose oxidase}}$ H₂O₂ +

D-glucono- δ -lactone

H₂O₂ + reduced chromagen $\xrightarrow{\text{peroxidase}}$ oxidized chromogen
+ H₂O

Chromagen

Glucostat reagent

4 N HCl

Dissolved chromagen and the glucostat reagent were mixed according to the package instructions and the volume was brought to 80 ml (macro method). Aliquots of 8 ml of this solution were added to 2 ml volumes in the standard, blank, and sample tubes at 45 sec intervals. The tubes were mixed and allowed to stand 10 min. At 45 sec intervals 1 drop 4 N HCl was added to each tube. The tubes were mixed and were allowed to stand at least 5 min. The absorbance was read at 420 nm in a Gilford 240 spectrophotometer; standards were glucose.

A procedure for deproteinization was necessary before the assay could be done successfully.

0.14 N NaOH

2% ZnSO₄·7H₂O

The standard, blank, and samples were treated with 1 ml of the NaOH solution and 1 ml of the Zn solution diluted 1:4 with distilled water, and after mixing, were centrifuged at 755 x g for 5 min. A sample of 2 ml of the supernatant was used for the glucostat test.

Cellulase Viscometric Assay (Thomas and Mullins 1969)

Carboxymethylcellulose substrate:

1.2% CMC

0.018 M citrate-NaOH buffer pH 5.0

0.05% merthiolate

distilled water to one liter

Portions of 5 ml of the substrate were added to size 300 Ostwald-Fenske Viscosity tubes and placed in a preheated 30°C water bath. A 1 ml sample of the enzyme solution or the appropriate blank was added and the resulting solution was mixed by gentle suction. The solution was then drawn by suction into a glass bulb near the top of the tube and the rate at which it fell back into the bottom tube was measured. Similar measurements were taken at time intervals. A cellulase unit is equal to the percent decrease in flow time per hr divided by 10%.

DMAB Assay (Reissig *et al.* 1955)

0.8 M KBO_4

p-dimethylaminobenzaldehyde (Sigma) diluted with 9 volumes of glacial acetic acid

A 0.1 ml aliquot of the KBO_4 solution was added to 0.5 ml of the sample, blank, and standard tubes and these were heated in a vigorously boiling water bath for 3 min. After cooling in tap water, 3 ml of the DMAB solution were added and the samples were mixed with a vortex and placed in a 36 to 38°C water bath for 20 min. After cooling in tap water the absorbance was read immediately at 544 nm on a Gilford 240 spectrophotometer. N-acetylglucosamine solutions were used as standards.

The preparation of KBO_4 solution is described by the following procedure:

0.9 M H_3BO_3

2 N KOH

A total of 35 ml of 0.9 M H_3BO_3 was stirred on a hot plate and 2 N KOH was added with stirring until the pH was raised to 9.2. The volume was brought to 50 ml which decreased the molarity to about 0.8 M.

Folin Test (Lowry *et al.* 1951)

0.5% CuSO₄
2% sodium tartrate
2% Na₂CO₃
Folin reagent
0.1 N NaOH

Blanks, standard, and sample volumes were all brought to 1 ml with 0.1 N NaOH (Reiskind 1970). A solution of 0.5 ml CuSO₄, 0.5 ml sodium tartrate, and 50 ml Na₂CO₃ was made and 1 ml of this was added at 45 sec intervals to the solutions to be tested which were mixed immediately with a vortex. After exactly 10 min 0.1 ml of the Folin reagent was added at 45 sec intervals and mixed immediately. The absorbance of the tubes was read 30 min later at 45 sec intervals at 750 nm on a Gilford 240 spectrophotometer against a 0.1 N blank. The protein standards were made with bovine serum albumen (Sigma).

BioRad Protein Assay (BioRad Technical Bulletin No. 1051)

To 0.1 ml blank, standard, and sample solutions was added 5.0 ml of diluted BioRad dye reagent (1 dye reagent: 4 distilled water using filtration to remove the precipitate) and the solutions were mixed on a vortex. They were allowed to stand for a minimum of 5 min and a maximum of 60 min, and their absorbance was read against the appropriate blank at 595 nm in a Gilford 240 spectrophotometer. Gamma globulin was used as a protein standard.

Lipase Assay (Bier 1955)

Tween 20
0.2 M sodium phosphate-citrate pH 6.2 buffer
0.02% phenol red
octyl alcohol
0.02 N NaOH

To 5 ml substrate solution (1 ml tween 20 + 2.0 ml 0.2 M buffer + 1.8 ml H_2O + 0.2 ml phenol red) is added 1 ml enzyme solution. The tubes were stoppered and placed in a 20°C water bath for 9 min. One drop octyl alcohol was added to prevent foaming. The solution was titrated with 0.02 N NaOH under a N_2 stream with stirring such that the end point was reached at 10 min after the initial addition of the enzyme to the substrate. Enzyme activity was determined by calculating the difference between the alkali consumed in the test solutions and the sum of the two blanks. A ml of titrant equals 100 lipase units. In the tests of lipase activity against various substrates, the reaction mixtures after 24 hr of incubation were titrated directly.

Carbazole Test (Bitter and Muir 1962)

0.025 M sodium tetraborate· $10H_2O$
concentrated H_2SO_4
Carbazole (Eastman Kodak)
Methanol, analytical grade

In screw-capped culture test tubes 5 ml of a solution of sodium tetraborate in concentrated sulfuric acid was cooled to 4°C, after which 1 ml sample, blank, or standard solution was layered onto the acid. The caps of the tubes were loosely screwed and the tubes were placed in an ice bath and shaken gently at first and then vigorously; the temperature was never allowed to exceed room temperature. The tubes were then placed for 10 min in a vigorously boiling water bath and then were cooled to room temperature. An aliquot of 0.2 ml carbazole (0.125% carbazole in methanol) reagent was added to the tubes which were shaken and heated in a boiling water bath for 15 more minutes. The tubes were then cooled to room temperature and their absorbance was read at 530 nm in a Gilford 240 spectrophotometer. The standard used was glucuronolactone.

Carbazole reagent is stable for 12 weeks if stored at 4°C in the dark.

Fiske-Subbarow Assay (Bartlett 1959)

0.22% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$
Fiske-Subbarow reagent (Sigma)

To the combusted material (initial volume not to exceed 2 ml) was added 4.6 ml of the molybdate solution and 0.2 ml of the Fiske-Subbarow reagent. The solution was mixed thoroughly and heated for 7 min in a boiling water bath with marbles covering the tubes. The absorbance of the cooled tubes was read at 830 nm on a Gilford 240 spectrophotometer. The standard was made from dilutions of a 1 mM solution of Na_2HPO_4 such that the final concentration of phosphorus in the assay mixture ranged from about 0.2 to 0.02 μM .

APPENDIX C PERIPHERAL STUDIES

Dry Weight Determination of Washed Mycelium

Dry weights of freshly harvested 48 hr washed mycelium were determined on material which had been dried in tared weighing pans *in vacuo* over desiccant for three days. Percent dry weight of fresh weight was determined. Percent dried wall of the dried weight was also calculated. Mycelial dry weight was estimated to be about 1.33% of the fresh weight of washed mycelium. Of this 51.3% or 0.682 gm is wall estimated from buffer-water washed material.

GLC Analyses of H₂SO₄ Hydrolysates of Unfractionated Walls

In general the results of the sulfuric acid hydrolysis of the wall were disappointing, mainly because none of the expected dissacharides appeared. The one interesting fact about these chromatograms (Fig. 23) was that a small amount of measurable glucosamine was found while only trace amounts of this sugar appeared in some of the HCl hydrolysates.

Congo Red Stain of the Wall and its Fractions

Congo red, a specific stain for cellulose, was used to determine the presence or absence of cellulose in the various wall fractions and in the wall itself. The acid-soluble fraction did not take up the stain at all while the wall itself was stained moderately. Cellulose I and II were stained heavily and both the alkali-soluble fraction and the insoluble residuum were stained moderately.

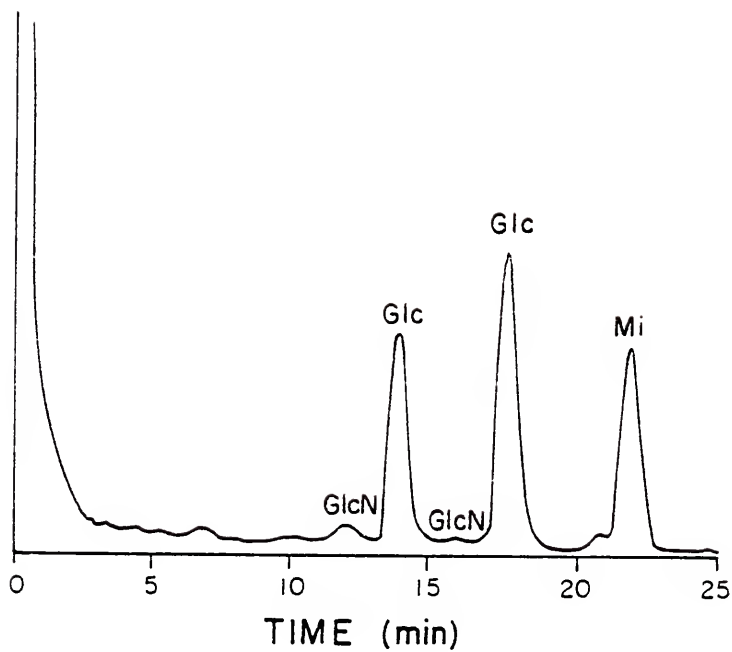


Figure 23. GLC of the TMS derivatives of the monosaccharides released by H_2SO_4 hydrolysis of the unfractionated wall. In addition to glucose small peaks of glucosamine are also seen.

Wall Width as Measured from Thin Section Micrographs

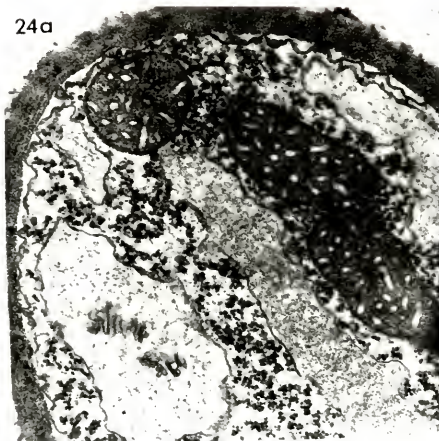
The width of the wall was measured from sections near the tip and those more distal (Fig. 24a and b). Sections of wall on each micrograph were divided into fifths and measurements were taken at each fifth. Examination of the micrographs (Figs. 24a and b) reveals that the wall is thicker in some areas than in others; it is not known if the added thickness is an artifact produced during the preparation of the sections or not. However, the measurements made of the wall width include the added thickness if that was the situation where the measurements were made. Widths were taken from three different micrographs of apical and subapical walls. The average width of the wall near the tip was $198.0 \text{ nm} \pm 32.15$ and the more distal $299.2 \text{ nm} \pm 37.58$. This is in partial agreement with Hill (Thesis 1978) who found the width to be $178 \text{ nm} \pm 100$.

Observations of Enzymatically Treated Material by Phase Microscopy

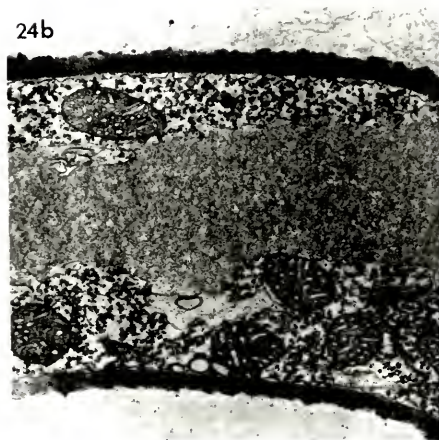
The material from which surface replicas were made was observed under phase microscopy. Because of the relatively low magnification used in these studies compared with that of the electron microscope, it was difficult to ascertain very accurately the effect of the enzymes. In general it can be said that the walls treated with single enzymes appeared normal. Those treated with the two enzyme sequence appeared a little thinner than those treated with one. The walls treated with the three enzyme sequence definitely appeared thinner and in some cases parts of the wall were missing and the characteristic tubular hyphal shape was destroyed.

Figure 24. Apical and subapical sections of an *A. ambisexualis* hypha. (a) Apical, X 25 500. (b) Subapical, X 13 300.

24a



24b



Observations of Hyphal Branching by Polarizing
Light Microscopy

As mentioned in the main body of this study observations of hyphae with polarized light demonstrated birefringence. However, birefringence was not seen in antheridial branches nor in the areas where branching occurred. Very strong birefringence was noted though in the corners between the parent hyphae and the branch points, as if the "softened" wall in the parent hyphae was pushed aside, possibly by turgor pressure, to allow the incipient branch to develop (Thomas 1970). Therefore, it seems in branching as if the cellulosic component of the wall is changed to accommodate the developing branch, but what happens to the matrix of the wall is not known. Is it softened, stretched or dissolved to make way for the new branch? Perhaps its function is to hold the wall together in the isolated areas of branch induction after cellulosic "softening."

REFERENCES

- Albersheim, P. 1974. The walls of growing plant cells. *Sci. Amer.* 80: 81-95.
- Albersheim, P., W. D. Bauer, K. Keegstra, and K. W. Talmadge. 1973. The structure of the walls of suspension-cultured Sycamore cells. *In* Biogenesis of plant cell wall polysaccharides. *Edited by* F. Loewus. Academic Press, N.Y. pp. 117-147.
- Alexopoulos, C. 1952. Introductory mycology. John Wiley and Sons, Inc., N.Y. 482 pp.
- Ames, B. N. 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. *J. Bacteriol.* 93: 833-843.
- Aronson, J. M. 1965. The cell wall. *In* The fungi, Vol. I. *Edited by* G. C. Ainsworth and A. S. Sussman. Academic Press, N.Y. pp. 49-76.
- Aronson, J. M., B. A. Cooper, and M. S. Fuller. 1967. Glucans of Oomycete cell walls. *Science*, 155: 332-335.
- Aronson, J. M., and M. S. Fuller. 1969. Cell wall structure of the marine fungus, *Atkinsiella dubia*. *Arch. Mikrobiol.* 68: 395-305.
- Aronson, J. M., and C. C. Lin. 1978. Hyphal wall chemistry of *Leptomitus lacteus*. *Mycologia*, 70: 363-369.
- Aronson, J. M., and L. Machlis. 1959. The chemical composition of the hyphal walls of the fungus *Allomyces*. *Amer. J. Bot.* 46: 292-300.
- Aronson, J. M., and R. D. Preston. 1960. An electronmicroscopic and x-ray analysis of the walls of selected lower Phycomycetes. *Proc. Roy. Soc. B*, 152: 346-352.
- Arsenault, G. P., K. Biemann, A. W. Barksdale, and T. C. McMorris. 1968. The structure of antheridiol, a sex hormone in *Achlya bisexualis*. *J. Amer. Chem. Soc.* 90: 5635-5636.
- Aspinall, G. O. 1973. Carbohydrate polymers of plant cell walls. *In* Biogenesis of plant cell wall polysaccharides. *Edited by* F. Loewus. Academic Press, N.Y. pp. 95-115.
- Bacon, J. S. D., B. D. Milne, I. F. Taylor, and D. M. Webley. 1965. Features of the cell wall structure of yeast revealed by the action of enzymes from a non-fruiting myxobacterium (*Cytophaga johnsonii*). *Biochem. J.* 95: 28c-30c.

- Barksdale, A. W. 1960. Interthallic sexual relations in *Achlya*, a genus of aquatic fungi. *Amer. J. Bot.* 47: 14-23.
- Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234: 466-468.
- Bartnicki-Garcia, S. 1966. Chemistry of hyphal walls of *Phytophthora*. *J. Gen. Microbiol.* 42: 57-69.
- Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Ann. Rev. Microbiol.* 22: 87-108.
- Bartnicki-Garcia, S. 1973. Fundamental aspects of hyphal morphogenesis. *Symp. Soc. Gen. Microbiol.* 23: 245-267.
- Bartnicki-Garcia, S., and E. Lippman. 1966. Liberation of protoplasts from the mycelium of *Phytophthora*. *J. Gen. Microbiol.* 42: 411-416.
- Bartnicki-Garcia, S., and E. Lippman. 1967. Enzymic digestion and glucan structure of hyphal walls of *Phytophthora cinnamomi*. *Biochim. Biophys. Acta*, 136: 533-543.
- Bartnicki-Garcia, S., and E. Lippman. 1972. The bursting tendency of hyphal tips of fungi: presumptive evidence for a delicate balance between wall synthesis and wall lysis in apical growth. *J. Gen. Microbiol.* 73: 487-500.
- Bartnicki-Garcia, S., and W. J. Nickerson. 1962. Isolation, composition, and structure of the cell walls of filamentous and yeast-like forms of *Mucor rouxii*. *Biochim. Biophys. Acta*, 58: 102-119.
- Bartnicki-Garcia, S., and E. Reyes. 1964. Chemistry of spore wall differentiation in *Mucor rouxii*. *Arch. Biochem. Biophys.* 108: 125-133.
- Bartnicki-Garcia, S., and E. Reyes. 1968. Polyuronides in the cell walls of *Mucor rouxii*. *Biochim. Biophys. Acta*, 170: 54-62.
- Bauer, W. D., K. W. Talmadge, K. Keegstra, and P. Albersheim. 1973. The structure of plant cell walls. II. The hemicellulose of the walls of suspension-cultured Sycamore cells. *Pl. Physiol.* 51: 174-187.
- Beakes, G. W., J. L. Gay. 1978. Light and electron microscopy of oospore maturation in *Saprolegnia furcata*. Part 2. Wall development. *Trans. Brit. Mycol. Soc.* 71: 25-36.
- Berg, B. 1978. Cellulose degradation and cellulase formation by *Phialophora malorum*. *Arch. Microbiol.* 118: 61-65.
- Bhatti, T., R. E. Chambers, and J. R. Clamp. 1970. The gas chromatographic properties of biologically important N-acetylglucosamine derivatives, monosaccharides, disaccharides, trisaccharides, tetrasaccharides, and pentasaccharides. *Biochem. Biophys. Acta*, 222: 339-347.

- Bier, M. 1955. Lipases. In *Methods in enzymology*, Vol. I. Edited by S. P. Colowick and N. O. Kaplan. Academic Press, N.Y. pp. 627-642.
- BioRad Technical Bulletin #1051. 1977. BioRad Laboratories. Richmond, California.
- Bishop, C. T. 1964. Gas-liquid chromatography of carbohydrate derivatives. *Adv. Carbohy. Chem.* 19: 95-147.
- Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4: 330-334.
- Block, R. J., E. L. Durrum, and G. Zweig. 1958. A manual of paper chromatography and paper electrophoresis. Academic Press, N.Y. 710 pp.
- Bobbitt, J. M. 1956. Periodate oxidation of carbohydrates. In *Advances of carbohydrate chemistry*, Vol. II. Edited by M. I. Wolfrom. Academic Press, N.Y. pp. 1-41.
- Bradley, D. E. 1965. Replica and shadowing techniques. In *Techniques for electron microscopy*, 2nd Edition. Edited by D. Kay. F. A. Davis Co., Philadelphia. pp. 96-152.
- Burnett, J. H. 1968. *Fundamentals of mycology*. Edward Arnold, Ltd., London. 548 pp.
- Cabib, E. 1975. Molecular aspects of yeast morphogenesis. *Ann. Rev. Microbiol.* 29: 191-214.
- Cameron, D. W., and I. E. P. Taylor. 1976. Quantitative microanalysis of cell walls of *Saprolegnia diolina* Humphrey and *Tremella mesenterica* Fries. *Biochim. Biophys. Acta*, 444: 212-222.
- Cardemil, L., and G. Pincheira. 1979. Characterization of the carbohydrate component of fraction I in the *Neurospora crassa* cell wall. *J. Bacteriol.* 137: 1067-1072.
- Clamp, J. R., T. Bhatti, and R. E. Chambers. 1971. The determination of carbohydrate in biological materials by gas-liquid chromatography. In *Methods of biochemical analysis*, Vol. 19. Edited by D. Glick. John Wiley and Sons, N.Y. pp. 229-344.
- Cole, G. T., Sekiya, R. Kasai, T. Kokoyama, and Y. Nozawa. 1979. Surface ultrastructure and chemical composition of the cell walls on conidial fungi. *Exp. Mycol.* 3: 132-156.
- Cooper, B. A., and J. M. Aronson. 1967. Cell wall structure of *Pythium debaryanum*. *Mycologia*, 59: 658-670.

- Cowling, E. B. 1963. Structural features of cellulose that influence its susceptibility to enzymatic hydrolysis. In *Advances in enzymic hydrolysis of cellulose and related materials*. Edited by E. T. Reese. Macmillan Company, N.Y. pp. 1-32.
- Crook, E. M., and I. R. Johnston. 1962. The qualitative analysis of the cell walls of selected species of fungi. *Biochem. J.* 82: 325-331.
- Datema, R., H. Van den Ende, and J. G. H. Wessels. 1977a. The hyphal wall of *Mucor mucedo*. I. Polyanionic polymers. *Eur. J. Biochem.* 80: 611-619.
- Datema, R., J. G. H. Wessels, and H. Van den Ende. 1977b. The hyphal wall of *Mucor mucedo*. 2. Hexosamine-containing polymers. *Eur. J. Biochem.* 80: 621-626.
- Davison, P. K., and R. Young. 1969. Gas chromatography of carbohydrates. The quantitative determination of the free sugars of plants as their trimethylsilyl ethers. *J. Chromatog.* 41: 12-21.
- Desjardins, P. R., M. C. Wang, and S. Bartnicki-Garcia. 1973. Electron microscopy of zoospores and cysts of *Phytophthora palmivora*: morphology and surface texture. *Arch. Mikrobiol.* 88: 61-70.
- deVries, O. M. H., and J. G. H. Wessels. 1972. Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. *J. Gen. Microbiol.* 73: 13-22.
- deVries, O. M. H., and J. G. H. Wessels. 1973a. Effectiveness of a lytic enzyme preparation from *Trichoderma viride* in releasing spheroplasts from fungi, particularly basidiomycetes. *Antonie van Leeuwenhoek*, 39: 397-400.
- deVries, O. M. H., and J. G. H. Wessels. 1973b. Release of protoplasts from *Schizophyllum commune* by combined action of purified β 1,3-glucanase and chitinase derived from *Trichoderma viride*. *J. Gen. Microbiol.* 76: 319-330.
- Dietrich, S. M. C. 1973. Carbohydrates from the hyphal walls of some Oomycetes. *Biochim. Biophys. Acta*, 313: 95-98.
- Dische, Z. 1962. General color reactions. In *Methods in carbohydrate chemistry*, Vol. I. Edited by R. L. Whistler and M. L. Wolfrom. Academic Press, N.Y. pp. 477-512.
- Dow, J. M., and P. H. Rubery. 1977. Chemical fractionation of the cell walls of mycelial and yeast-like forms of *Mucor rouxii*: a comparative study of the polysaccharide and glycoprotein components. *J. Gen. Microbiol.* 99: 29-41.
- Dyer, J. R. 1956. Use of periodate oxidations in biochemical analysis. In *Methods of biochemical analysis*, Vol. III. Edited by D. Glick. Interscience Publishers, Inc., N.Y. pp. 111-152.

- Edwards, J. A., J. S. Mills, J. Sundeen, and J. H. Fried. 1969. The synthesis of the fungal sex hormone antheridiol. *J. Amer. Chem. Soc.* 91: 1248-1249.
- Eveleigh, D. E., J. H. Sietsma, and R. H. Haskins. 1968. The involvement of cellulase and laminarinase in the formation of *Pythium* protoplasts. *J. Gen. Microbiol.* 52: 89-97.
- Fan, L. T., Y. H. Lee, and D. H. Beardmore. 1980. Mechanism of the enzymatic hydrolysis of cellulose: effects of major structural features of cellulose on enzymatic hydrolysis. *Biotech. Bioeng.* 22: 177-199.
- Farkas, V. 1979. Biosynthesis of cell walls of fungi. *Microbiol. Rev.* 43: 117-144.
- Fèvre, M. 1977. Subcellular localization of glucanase and cellulase in *Saprolegnia monoica* Pringsheim. *J. Gen. Microbiol.* 103: 287-295.
- Fuller, M. S., and I. Barshad. 1960. Chitin and cellulose in the cell walls of *Rhizidiomyces* sp. *Amer. J. Bot.* 47: 105-109.
- Gancedo, J. M., C. Gancedo, and C. Asensio. 1966. Uronic acids in fungal cell walls. *Biochem. Zeitsch.* 346: 328-332.
- Gander, J. E. 1974. Fungal cell wall glycoproteins and peptidopolysaccharides. *Ann. Rev. Microbiol.* 28: 103-119.
- Ghose, T., and V. S. Bisaria. 1979. Studies in the mechanism of enzymatic hydrolysis of cellulosic substances. *Biotech. Bioeng.* 21: 131-146.
- Goldstein, I. J., G. W. Hay, B. A. Lewis, and F. Smith. 1965. Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. *In Methods in carbohydrate chemistry*, Vol. V. Edited by R. L. Whistler, B. C. Miller, and J. N. Wolfrom. Academic Press, N.Y. pp. 361-370.
- Green, J. W. 1963. Drying and reactivity of cellulose. *In Methods of carbohydrate chemistry*, Vol. III. Edited by R. L. Whistler and M. L. Wolfrom. Academic Press, N.Y. pp. 100-101.
- Hawes, C. R. 1979. Conidium ultrastructure and wall architecture in the chalaral state of *Ceratoecystis adiposa*. *Trans. Brit. Mycol. Soc.* 72: 177-187.
- Hay, G. W., B. A. Lewis, and F. Smith. 1965. Periodate oxidation of polysaccharides: general procedures. *In Methods of carbohydrate chemistry*, Vol. V. Edited by R. L. Whistler, B. C. Miller, and J. N. Wolfrom. Academic Press, N.Y. pp. 357-360.
- Heath, I. B., J. L. Gay, and A. D. Greenwood. 1971. Cell wall formation in the Saprolegniales: cytoplasmic vesicles underlying developing walls. *J. Gen. Microbiol.* 65: 225-232.

- Hegnauer, H., and H. R. Hohl. 1978. Cell wall architecture of sporangia, chlamydospores, oogonia, and oospores in *Phytophthora*. Exp. Mycol. 2: 216-233.
- Hill, T. W. 1978. Isolation and characterization of vesicles involved in hyphal tip growth of *Achlya*. Dissertation. University of Florida.
- Hirayama, T., H. Nagayama, and K. Matsuda. 1976. Studies on cellulases of a phytopathogenic fungus, *Pyricularia oryzae* Cavara. III. Multiplicity of β -glucosidase, and purification and properties of a second component. J. Biochem. 85: 591-599.
- Horowitz, D. K., and P. J. Russell. 1974. Hormone-induced differentiation of antheridial branches in *Achlya ambisexualis*: dependence on ribonucleic acid synthesis. Can. J. Microbiol. 20: 977-980.
- Hunsley, D., and J. H. Burnett. 1968. Dimensions of microfibrillar elements in fungal walls. Nature, 218: 462-463.
- Hunsley, D., and J. H. Burnett. 1970. The ultrastructural architecture of the walls of some hyphal fungi. J. Gen. Microbiol. 62: 203-218.
- Ishizuka, I., N. Ueta, and T. Yamakawa. 1966. Gas chromatographic studies of microbial components. II. Carbohydrate and fatty acid constitution of the family Micrococcaceae. Jap. J. Exp. Med. 36: 73-83.
- Jayme, G., and F. Lang. 1963. Cellulose solvents. In Methods in carbohydrate chemistry, Vol. III. Edited by R. L. Whistler and M. L. Wolfrom. Academic Press, N.Y. pp. 75-83.
- Jayme, G., and K. Neuschaffer. 1957. Tri-(en)-cadmium-hydroxyd als neues farbloses, wäBriges Lösungsmittel für cellulose. Die Naturwissenschaften, 44: 62-63.
- Kane, B. E. 1971. Regulatory mechanisms in cellulase synthesis during hyphal morphogenesis in the water mold *Achlya*. Dissertation. University of Florida.
- Kane, B. E., J. B. Reiskind, and J. T. Mullins. 1973. Hormonal control of sexual morphogenesis in *Achlya*: dependence on protein and ribonucleic acid syntheses. Science, 180: 1192-1193.
- Kanfer, J., and E. P. Kennedy. 1963. Metabolism and function of bacterial lipids. I. Metabolism of phospholipids in *Escherichia coli* B*. J. Biol. Chem. 238: 2919-2922.
- Keegstra, K., K. W. Talmadge, W. D. Bauer, and P. Albersheim. 1973. The structure of plant cell walls. III. A model of the walls of suspension-cultured Sycamore cells based on the interconnections of the macromolecular components. Pl. Physiol. 51: 188-196.

- Kidby, D. K., and R. Davies. 1970. Invertase and disulphide bridges in the yeast wall. *J. Gen. Microbiol.* 61: 327-333.
- Korn, E. D., and D. H. Northcote. 1960. Physical and chemical properties of polysaccharides and glycoproteins of the yeast-cell wall. *Biochem. J.* 75: 12-17.
- Kowkabany, G. N. 1954. Paper chromatography of carbohydrates and related compounds. In *Advances in carbohydrate chemistry*, Vol. 9. Edited by M. L. Wolfrom. Academic Press, N.Y. pp. 303-353.
- Ladisch, M. R., C. M. Ladisch, and G. T. Tsao. 1978. Cellulose to sugars: new path gives quantitative yield. *Science*, 201: 743-745.
- Lampen, J. O. 1968. External enzymes of yeast: their nature and formation. *Antonie van Leeuwenhoek*, 34: 1-18.
- Lampert, D. T. A. 1965. The protein component of primary cell walls. In *Advances in botanical research*. Edited by R. D. Preston. Academic Press, N.Y. pp. 151-218.
- Lampert, D. T. A. 1967. Hydroxyproline-o-glycosidic linkage of the plant cell wall glycoprotein extensin. *Nature*, 216: 1322-1324.
- Lampert, D. T. A. 1969. The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymic degradation of primary cell walls. *Biochem.* 8: 1155-1163.
- Lampert, D. T. A. 1970. Cell wall metabolism. *Ann. Rev. Pl. Physiol.* 21: 255-270.
- Lampert, D. T. A. 1973. The glycopeptide linkages of extensin: O-D-galactosyl serine and O-L-arabinosyl hydroxyproline. In *Biogenesis of plant cell wall polysaccharides*. Edited by F. Loewus. Academic Press, N.Y. pp. 147-164.
- Lampert, D. T. A., and D. H. Northcote. 1960. Hydroxyproline in primary cell walls of higher plants. *Nature*, 188: 665-666.
- Lehninger, A. L. 1975. *Biochemistry*, 2nd edition. Worth Publishers, Inc., N.Y. 1104 pp.
- Leppard, G. G., L. C. Sowden, and J. R. Colvin. 1975. Nascent stage of cellulose biosynthesis. *Science*, 189: 1094-1095.
- Letourneau, D. R., J. M. Deven, and M. S. Manocha. 1976. Structure and composition of the cell wall of *Choanephora cucurbitarum*. *Can. J. Microbiol.* 22: 486-494.
- Lin, C. C., and J. M. Aronson. 1970. Chitin and cellulose in the cell walls of the Oomycete, *Apodachlya* sp. *Arch. Mikrobiol.* 72: 111-114.

- Lin, C. C., C. Sicher, Jr., and J. M. Aronson. 1976. Hyphal wall chemistry in *Apodachlya*. Arch. Microbiol. 108: 85-91.
- Lippman, E., D. C. Erwin, and S. Bartnicki-Garcia. 1974. Isolation and chemical composition of oospore-oogonium walls of *Phytophthora megasperma* var. *sojae*. J. Gen. Microbiol. 80: 131-141.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
- Mahadevan, P. R., and E. L. Tatum. 1965. Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. J. Bacteriol. 90: 1073-1081.
- Mahadevan, P. R., and E. L. Tatum. 1967. Localization of structural polymers in the cell wall of *Neurospora crassa*. J. Cell. Biol. 35: 295-302.
- Manners, D. J., A. J. Masson, and J. C. Patterson. 1973a. The structure of a $\beta(1\rightarrow3)$ -D-glucan from yeast cell walls. Biochem. J. 135: 19-30.
- Manners, D. J., A. J. Masson, J. C. Patterson, H. Björndal, and B. Lindberg. 1973b. The structure of a $\beta(1\rightarrow6)$ -D-glucan from yeast cell walls. Biochem. J. 135: 31-36.
- McMorris, T. C., and A. W. Barksdale. 1967. Isolation of a sex hormone from the water mold *Achlya bisexualis*. Nature, 215: 320-321.
- McMorris, T. C., R. Seshadri, G. R. Weihe, G. P. Arsenault, and A. W. Barksdale. 1975. Structures of oogoniol-1, -2, and -3, steroidal sex hormones of the water mold, *Achlya*. J. Amer. Chem. Soc. 97: 2544-2545.
- McNair, H. M., and E. J. Bonelli. 1968. Basic gas chromatography. Varian Aerography, Walnut Creek, Cal. 306 pp.
- Mendoza, C. G., J. A. Leal, and M. Novaes-Ledieu. 1979. Studies of the spore walls of *Agaricus bisporus* and *Agaricus campestris*. Can. J. Microbiol. 25: 32-39.
- Mitchell, R., and N. Sabar. 1966. Hyphal cell wall structure of two species of *Pythium*. Can. J. Microbiol. 12: 471-475.
- Miyazaki, T., M. Yamada, and T. Ohno. 1974. Isolation and structure of $\alpha 1,3$ linked glucan from hyphal wall of *Phytophthora infestans*. Chem. Pharm. Bull. 22: 1666-1669.
- Mollenhauer, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technology, 39: 111-114.

- Monro, J. A., R. W. Bailey, and D. Penny. 1974. Cell wall hydroxy-proline-polysaccharide associations in *Lupinus* hypocotyls. *Phytochemistry*, 13: 375-382.
- Monro, J. A., D. Penny, and R. W. Bailey. 1976. The organization and growth of primary cell walls of lupin hypocotyl. *Phytochemistry*, 15: 1193-1198.
- Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science*, 197: 254-255.
- Mullins, J. T. 1979. A freeze-fracture study of hormone-induced branching in the fungus *Achlya*. *Tissue and Cell*, 11: 585-595.
- Mullins, J. T., and A. W. Barksdale. 1965. Parasitism of the chytrid *Dictyomorpha dioica*. *Mycologia*, 57: 352-359.
- Mullins, J. T., and E. A. Ellis. 1974. Sexual morphogenesis in *Achlya*: ultrastructural basis for the hormonal induction of antheridial hyphae. *Proc. Nat. Acad. Sci. U.S.A.* 71: 1347-1350.
- Muzzarelli, R. A. A. 1977. Chitin. Pergamon Press, N.Y. 309 pp.
- Northcote, D. H. 1963. The nature of plant cell surfaces. In *The structure and function of the membranes and surfaces of cells*. Biochem. Soc. Symp. 22. Edited by D. J. Bell and J. K. Grant. Cambridge University Press, Cambridge. pp. 105-125.
- Novaes-Ledieu, M., and A. Jiménez-Martínez. 1969. The structure of cell walls of Phycomycetes. *J. Gen. Microbiol.* 54: 407-415.
- Novaes-Ledieu, M., A. Jiménez-Martínez, and J. R. Villaneuva. 1967. Chemical composition of hyphal wall of Phycomycetes. *J. Gen. Microbiol.* 47: 237-245.
- Nyhlén, L., and T. Unestam. 1978. Cyst and germ tube wall structure in *Aphanomyces astaci*, Oomycetes. *Can. J. Microbiol.* 24: 1293-1299.
- Okazaki, M., and M. Moo-Young. 1978. Kinetics of enzymatic hydrolysis of cellulose: analytical description of a mechanistic model. *Biotech. Bioeng.* 20: 637-663.
- Pao, V. M., and J. M. Aronson. 1970. Cell wall structure of *Sapromyces elongatus*. *Mycologia*, 62: 531-541.
- Parker, B. C., R. D. Preston, and G. E. Fogg. 1963. Studies of the structure and chemical composition of the cell walls of Vaucheriaceae and Saprolegniaceae. *Proc. Roy. Soc. B, London*, 158: 435-445.
- Partridge, S. M. 1944. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature*, 164: 443.

- Pease, O. C. 1964. Histological techniques for electron microscopy. 2nd Edition. Academic Press, N.Y. 381 pp.
- Pengra, R. M., M. A. Cole, and M. Alexander. 1969. Cell walls and lysis of *Mortierella parvispora* hyphae. J. Bacteriol. 97: 1056-1061.
- Pierce, A. E. 1968. Silylation of organic compounds. Pierce Chemical Company, Rockford, Illinois. 487 pp.
- Potgieter, J. H., and M. Alexander. 1965. Polysaccharide components of *Neurospora crassa* hyphal walls. Can. J. Microbiol. 11: 122-125.
- Prakasam, V. R., and J. Azariah. 1975. An optimum pH for the demonstration of chitin in *Periplaneta americana* using Lugol's iodine. Acta histochem. 53: 238-240.
- Preston, R. D. 1974a. The physical biology of plant cell walls. Chapman and Hall, London. 491 pp.
- Preston, R. D. 1974b. Plant cell walls. In Dynamic aspects of plant ultrastructure. Edited by A. W. Robards. McGraw-Hill Book Company, N.Y. pp. 256-309.
- Preston, R. D. 1979. Polysaccharide conformation and cell wall function. Ann. Rev. Physiol. 30: 55-78.
- Raper, J. R. 1939a. Role of hormones in the sexual reaction of heterothallic *Achlya*. Science, NS. 89: 321-322.
- Raper, J. R. 1939b. Sexual hormones in *Achlya*. I. Indicative evidence for a hormonal coordinating mechanism. Amer. J. Bot. 26: 639-650.
- Raper, J. R. 1940. Sexual hormones in *Achlya*. II. Distance reactions, conclusive evidence for a hormonal coordinating mechanism. Amer. J. Bot. 27: 162-173.
- Rees, D. A. 1977. Polysaccharide shapes. John Wiley and Sons, N.Y. 80 pp.
- Reese, E. T., and M. Mandels. 1963a. Enzymic hydrolysis of β -glucans. In Advances of enzymic hydrolysis of cellulose and related materials. Edited by E. T. Reese. Macmillan Company, N.Y. pp. 197-234.
- Reese, E. T., and M. Mandels. 1963b. Enzymic hydrolysis of cellulose and its derivatives. In Methods of carbohydrate chemistry, Vol. III. Edited by R. L. Whistler and M. L. Wolfrom. Academic Press, N.Y. pp. 139-143.
- Reiskind, J. B. 1970. The role of translation in the hormonal induction of sexual morphogenesis in *Achlya*. Thesis. University of Florida.
- Reissig, J. L., J. L. Strominger, and L. F. Leloir. 1955. A modified colorimetric method for the estimation of N-acetyl amino sugars. J. Biol. Chem. 217: 959-966.

- Rosenberger, R. F. 1976. The cell wall. In *The filamentous fungi*, Vol. 2. Edited by J. E. Smith and D. R. Berry. John Wiley and Sons, N.Y. pp. 328-344.
- Ruiz-Herrera, J. 1967. Chemical components of the cell wall of *Aspergillus* species. Arch. Biochem. Biophys. 122: 118-125.
- Sasaki, T., and T. Tanaka, N. Naki, Y. Sato, and K. Kainuma. 1979. Correlation between x-ray diffraction measurements of cellulose crystalline structure and the susceptibility to microbial cellulase. Biotech. Bioeng. 21: 1031-1043.
- Sietsma, J. H., D. E. Eveleigh, and R. H. Haskins. 1968. The purification of cellulase and exo-laminarinase and their role in the formation of *Pythium* sp. protoplasts. Antonie van Leeuwenhoek, 34: 331-340.
- Sietsma, J. H., D. E. Eveleigh, and R. H. Haskins. 1969. Cell wall composition and protoplast formation of some Oomycete species. Biochim. Biophys. Acta, 184: 306-317.
- Sietsma, J. H., J. J. Child, L. R. Nesbitt, and R. H. Haskins. 1975. Chemistry and ultrastructure of the hyphal walls of *Pythium acanthicum*. J. Gen. Microbiol. 86: 29-38.
- Sietsma, J. H., and J. G. H. Wessels. 1977. Chemical analysis of the hyphal wall of *Schizophyllum commune*. Biochim. Biophys. Acta, 496: 225-239.
- Sietsma, J. H., and J. G. H. Wessels. 1979. Evidence for covalent linkages between chitin and β -glucan in a fungal wall. J. Gen. Microbiol. 114: 99-108.
- Skujins, J. J., H. J. Potgieter, and M. Alexander. 1965. Dissolution of fungal cell walls by a Streptomyces chitinase and β -(1 $\rightarrow$ 3) glucanase. Arch. Biochem. Biophys. 111: 358-364.
- Smith, F., and R. Montgomery. 1956. End group analysis of polysaccharides. In *Methods of biochemical analysis*, Vol. III. Edited by D. Glick. Interscience Publishers, Inc., N.Y. pp. 153-212.
- Sturgeon, R. J. 1974. Chemical and biochemical aspects of fungal cell walls. In *Plant carbohydrate biochemistry*. Edited by J. B. Pridham. Academic Press, N.Y. pp. 219-233.
- Sweeley, C. C., R. Bentley, M. Makita, and W. W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Amer. Chem. Soc. 85: 2497-2507.
- Talmadge, K. W., K. Keegstra, W. D. Bauer, and P. Albersheim. 1973. The structure of plant cell walls. I. The macromolecular components of the walls of suspension-cultured Sycamore cells with a detailed analysis of the pectic polysaccharides. Pl. Physiol. 51: 158-173.

- Taylor, I. E. P., and D. S. Cameron. 1973. Preparation and quantitative analysis of fungal cell walls: strategy and tactics. *Ann. Rev. Microbiol.* 27: 243-257.
- Thomas, D. des S. 1970. Effects of water stress on induction and secretion of cellulase and on branching in the water mold *Achlya ambisexualis*. *Can. J. Bot.* 48: 977-979.
- Thomas, D. des S., and J. T. Mullins. 1969. Cellulase induction and wall extension in the water mold *Achlya ambisexualis*. *Physiol. Plant.* 22: 347-353.
- Timberlake, W. E. 1976. Alterations in RNA and protein synthesis associated with steroid hormone-induced sexual morphogenesis in the water mold *Achlya*. *Devel. Biol.* 51: 202-214.
- Tokunaga, J., and S. Bartnicki-Garcia. 1971. Structure and differentiation of the cell wall of *Phytophthora palmivora*: cysts, hyphae and sporangia. *Arch. Mikrobiol.* 79: 293-310.
- Tracey, M. V. 1955. Chitin. In *Modern methods of plant analysis*, Vol. II. Edited by K. Paech and M. V. Tracey. Springer-Verlag, Berlin. pp. 264-274.
- Tristram, G. R., and R. H. Smith. 1963. The amino acid composition of some purified proteins. In *Advances in protein chemistry*, Vol. 18. Edited by C. B. Anfinsen, Jr., M. L. Anson, and J. T. Edsall. Academic Press, N.Y. pp. 227-318.
- Van der Valk, P., and J. G. H. Wessels. 1977. Light and electron microscopic autoradiography of cell-wall regeneration by *Schizophyllum commune* protoplasts. *Acta Bot. Neerl.* 26: 43-52.
- Wang, H. S., and H. B. LeJohn. 1974. Analogy and homology of the dehydrogenases of Oomycetes. I. Regulation of glutamic dehydrogenases and isozyme patterns. *Can. J. Microbiol.* 20: 567-574.
- Weijman, A. C. M. 1979. Cell-wall composition and taxonomy of *Cephaloscyus fragrans* and some Ophiostomataceae. *Antonie van Leeuwenhoek*, 42: 315-324.
- Wessels, J. G. H., D. R. Kreger, R. Marchant, B. A. Regensburg, and O. M. H. de Vries. 1972. Chemical and morphological characterization of the hyphal wall surface of the Basidiomycete *Schizophyllum commune*. *Biochim. Biophys. Acta*, 273: 346-358.
- Whitaker, D. R. 1970. Cellulases. In *The enzymes*, Vol. II, 3rd edition. Edited by P. D. Boyer. Academic Press, N.Y. pp. 273-290.
- White, A., P. Handler, and E. L. Smith. 1964. Principles of biochemistry. McGraw-Hill Book Company, N.Y. 1106 pp.

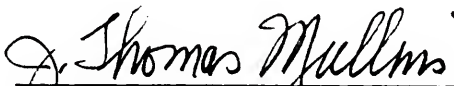
- Whitehouse, M. W., A. E. Bresler, and E. Staple. 1958. The use of iodine for the detection of lipids. *J. Chromatog.* 1: 385-386.
- Wolfe, S. L. 1972. *Biology of the cell*. Wadsworth Publishing Company, Inc., Belmont, California. 545 pp.
- Worthington Glucostat Reagent Set, Worthington Biochemical Corporation, Freehold, New Jersey.
- Wrathall, C. R., and E. L. Tatum. 1973. The peptides of the hyphal wall of *Neurospora crassa*. *J. Gen. Microbiol.* 78: 139-153.
- Yamada, M., and T. Miyazaki. 1976. Ultrastructure and chemical analysis of the cell wall of *Pythium debaryanum*. *Japan J. Microbiol.* 20: 83-91.
- Yamakawa, T., and N. Ueta. 1964a. Gas-liquid chromatography of carbohydrates. *Japan J. Exp. Med.* 34: 37-51.
- Yamakawa, T., and N. Ueta. 1964b. Gaschromatographic studies of microbial components. I. Carbohydrate and fatty acid constitution of *Neisseria*. *Jap. J. Exp. Med.* 34: 361-374.
- Youatt, J. 1977. Chemical nature of *Allomyces* walls. *Trans. Brit. Mycol. Soc.* 69: 187-190.
- Young, T. W. K. 1970. Arrangement of microfibrils in walls of aerial hyphae of *Linderina*. *Trans. Brit. Mycol. Soc.* 55: 29-35.
- Zanetta, J. P., W. C. Brechenridge, and G. Vincendon. 1972. Analysis of monosaccharides by gas-liquid chromatography of the O-methyl glycosides as trifluoroacetate derivatives. Application to glycoproteins and glycolipids. *J. Chromatog.* 69: 291-304.
- Zevenhuisen, L. P. T. M., and S. Bartnicki-Garcia. 1969. Chemical structure of the insoluble hyphal wall glucan of *Phytophthora cinnamomi*. *Biochem.* 8: 1496-1502.

BIOGRAPHICAL SKETCH

Julia Barth Reiskind was born in Hackensack, New Jersey, on June 26, 1941. She attended the public schools in Ridgewood, New Jersey, and graduated from high school in 1959. She received a Bachelor of Arts degree from Goucher College, Towson, Maryland, in biological sciences in 1963. She then worked as a Research Assistant in the laboratory of John R. Raper, Harvard University, Cambridge, Massachusetts, until 1967. She enrolled in graduate school at the University of Florida in 1968 and received a Master of Science in botany in 1970. She began her pursuit of the Ph.D. in botany in 1973 as a part-time student and has been working toward that end since that time. She is a member of Phi Kappa Phi Honorary Society, Sigma Xi, the Mycological Society of America, and the American Society of Plant Physiologists.

She is married to Dr. Jonathan Reiskind and has two children, Julia Alexandra and Michael Hay, ages nine and six, respectively.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



J. Thomas Mullins, Chairman
Professor of Botany

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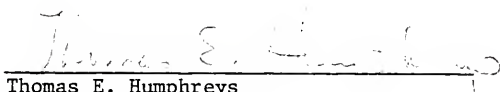
Mildred M. Griffith
Professor Emeritus of Botany

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